



Henrique da Silva Ferrão Nascimento

Erythrocyte membrane profile in obese children and adolescents

**Dissertação do 2º Ciclo de Estudos Conducente ao Grau de Mestre em
Análises Clínicas**

Trabalho realizado sobre a orientação dos professores:

Alice Santos Silva

Luís Filipe Barbosa Amado Belo

Outubro 2011

**É AUTORIZADA A REPRODUÇÃO INTEGRAL DESTA DISSERTAÇÃO APENAS
PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO ESCRITA DO
INTERESSADO QUE A TAL SE COMPROMETE.**

Agradecimentos

Agradeço a todos o apoio que tive na execução desta tese.

Aos meus orientadores, Professora Doutora Alice Santos Silva e Professor Doutor Luís Belo pela paciência, apoio e acompanhamento constante que me deram.

A Professora Doutora Susana Casal por todo o apoio, quer prático, quer teórico, trazendo uma enorme mais valia a esta tese.

Aos meus colegas de laboratório, particularmente ao João Fernandes, a Sandra Ribeiro, a Filomena Silva, a Susana Rocha, a Ana Borges, ao Elísio Vieira e a Susana Coimbra pela ajuda, quer a nível laboratorial, quer a nível teórico. Devo agradecer igualmente a eles, e aos demais colegas de laboratório, Bruno Fonseca, Cristina Amaral, David Pereira, Cristina Catarino e Mariana Costa pelo companheirismo e amizade que tornam todo o trabalho mais fácil e agradável.

A minha família, dos dois lados do Atlântico, e a minha namorada, agradeço pelo apoio e compreensão neste últimos 2 anos de estudo. A eles peço, também, desculpas pelo mau aproveitamento em particular deste último verão, em que passei a maior parte dos dias de sol sentando em frente ao computador.

A Dra. Carla Rego e a Dra. Helena Mansilha gostaria de agradecer todo o apoio e interesse que demonstraram para este trabalho desde o princípio, principalmente na selecção e avaliação clínica dos participantes.

Aos membros do Serviço de Análises Clínicas do Hospital de Crianças Maria Pia, a equipe de enfermagem da Pediatria do Hospital de São João e ao Serviço de Análises Clínicas da Faculdade de Farmácia da Universidade do Porto, em particular a Bárbara Duarte, agradeço a disponibilidade e o apoio dado com as colheitas dos participantes e parte da sua análise.

Resumo

A prevalência da obesidade está crescendo em todo o mundo, sendo este aumento particularmente notório na infância e adolescência. Os alimentos ricos em calorias e o estilo de vida sedentário são duas das características do estilo de vida moderno, que pode estar por de trás desta pandemia. A obesidade pediátrica é particularmente importante em nosso país: uma alta prevalência de sobrepeso / obesidade (30%) tem sido descrita em crianças e adolescentes portugueses, em comparação com seus pares de outros países europeus.

A obesidade tem sido associada a várias outras doenças, como hipertensão, diabetes mellitus tipo 2, síndrome metabólica e doenças cardiovasculares. Além disso, a inflamação é conhecida por desempenhar um papel vital nessas doenças, ou seja, na iniciação e progressão da doença arteriosclerótica vascular, na resistência à insulina, e dislipidemia. Mais recentemente, a obesidade tem sido proposta como um estado de baixo grau inflamatório crônico. De fato, O tecido adiposo é uma importante fonte de várias substâncias que estão ligadas à resposta inflamatória e à imunidade - as adipocinas.

As dietas de indivíduos obesos são, geralmente, ricos em gorduras saturadas e ácidos gordos (AG) trans, e pobres em AG polinsaturados (AGPI), especialmente, em AGPI do tipo ω 3. Este tipo de hábitos alimentares causa alterações endógenas no metabolismo lipídico, e pode levar a mudanças na constituição em AG em diferentes tecidos do corpo.

A composição lipídica das membranas celulares é importante para as propriedades reológicas e físico-químicas das células, influenciando a actividade dos canais protéicos e das bombas da membrana, assim como de transportadores e receptores membranares. Deste modo, o perfil lipídico da membrana é importante na modulação da sinalização celular e de várias funções biológicas da célula.

Os lípidos da membrana dos glóbulos vermelhos estão em equilíbrio constante com lípidos e lipoproteínas plasmáticos. Apesar dos níveis dos lípidos plasmáticos serem altamente influenciados pelo estado de jejum, os níveis e tipo de lípidos da membrana eritrocitária reflectem o equilíbrio lipídico por períodos mais longos.

Os glóbulos vermelhos (GV), por tratarem-se de células não-nucleadas, apresentam uma capacidade de biossíntese e mecanismos de defesa muito limitados. Portanto, quando

expostos a stress físico e / ou químico, o eritrócito sofre e acumula o dano imposta por aquelas fontes durante sua vida em circulação. Em condições de stress oxidativo a membrana do eritrócito, pode sofrer danos em seus lípidos e proteínas. Deste modo, o eritrócito é um bom modelo para estudar os danos oxidativos dos lípidos e proteínas que ocorrem em estados pró-inflamatórios e oxidativas, e também pode fornecer um importante modelo para estudar o impacto dos hábitos alimentares na composição dos lípidos e proteínas das membranas celulares.

O objectivo deste estudo foi analisar o impacto da obesidade no perfil lipídico, no metabolismo da glicose e na inflamação, bem como o impacto da obesidade, e das alterações a ela associadas, na composição da membrana do GV.

Foram estudadas 34 crianças e adolescentes obesos [15 (44,1%), com idade média de 14,1 anos (8-17)] do Hospital S. João e do Hospital Infantil Maria Pia. O grupo total foi dividido de acordo com o percentil do índice de massa corporal (IMC) em 17 obesos, 8 sobre-pesos e 9 controles. Obesidade foi definida como um IMC superior ao percentil 95, ajustados para idade e sexo, segundo " gráficos de crescimento do Centro de Controle de Doenças de 2000". Sobrepeso foi considerado para os percentis de IMC igual ou superior a 85 e inferior a 95; e controles quando o IMC era inferior ao percentil 85, ajustado para sexo e idade. Os três grupos estavam equilibrados para a idade, sexo e estágio de maturação sexual de Tanner.

Foram determinados os níveis circulantes de triglicerídeos, colesterol, colesterol de lipoproteína de alta densidade, colesterol de lipoproteína de baixa densidade, lipoproteína (a), apolipoproteína A, apolipoproteína B, proteína C-reativa, glicose e insulina. Um estudo hematológico básico foi realizado. A membrana eritrocitária foi estudada com a determinação de marcadores de lesão eritrocitária: hemoglobina ligada a membrana, carbonilação proteica, peroxidação lipídica e perfil de banda 3 – agregados de alto peso molecular, monómeros e fragmentos proteolíticos. O perfil de ácidos gordos da membrana foi também determinado.

Os indivíduos obesos apresentaram, quando comparados com os controlos, alterações para um perfil lipídico mais aterogénico, um aumento da resistência à insulina e da inflamação. Assim, houve um aumento geral dos marcadores de risco de doença cardiovascular (DCV). Nenhuma diferença significativa foi encontrada no eritrograma ou nos marcadores de lesão eritrocitária.

Quanto ao perfil de AG da membrana eritrocitária, os AG insaturados apresentaram uma tendência para o aumento, enquanto os AG saturados mostraram uma tendência para diminuir com a obesidade. Apesar disso, o AG beénico ácido (22:0) apresentou um aumento significativo nos obesos, em comparação com os controlos. Uma proporção crescente de 20:0, 18:3n3, 20:3n6e 22:4n6 foram encontrados para indivíduos com sobrepeso e obesos, em relação aos controlos. Estes AG, que aumentaram com a obesidade, apresentaram as associações mais significativas com os marcadores de DVC estudados e que estão alterados com a obesidade na nossa população.

Mais estudos são necessários para esclarecer as associações entre as alterações do perfil de AG da membrana eritrocitária e os marcadores de risco de DCV. Um estudo envolvendo mais participantes poderia ajudar a esclarecer algumas tendências observadas. Quanto à análise da membrana lipídica, algumas abordagens interessantes poderiam ser feitas, como analisar separadamente os AG ligados a fosfolípidos e ésteres de colesterol, ou analisar individualmente cada folheto da membrana plasmática (interno e externo). Além disso, a optimização da técnica de separação e identificação dos AG seria fundamental, pois no presente estudo não pudemos avaliar AG importante, como por exemplo o EPA (ácido eicosapentaenoico - 20:5n3) e GLA (ácido gama linolénico - 18:3 n6), devido a limitações técnicas.

Palavras-chave: Obesidade, eritrócitos, inflamação, stress oxidativo, dislipidemia, resistência à insulina. síndrome metabólica, metabolismo dos ácidos gordos.

Abstract

The prevalence of obesity is growing worldwide and in childhood the increase is particularly striking. The caloric rich foods and the reduced physical exercise practice are two of characteristics of the modern lifestyle that may underlie this pandemia.

Childhood obesity is particularly important in our country, as a high prevalence of overweight/obesity (over 30%) has been reported for Portuguese children, as compared to other European countries.

Obesity has been associated with several other diseases, such as hypertension, type 2 diabetes mellitus, metabolic syndrome and cardiovascular diseases. Moreover, inflammation is known to play a vital role in those diseases, namely, in the initiation and progression of the atherosclerotic vascular disease, in insulin resistance, and in dyslipidemia. More recently, obesity has been proposed as a chronic low grade inflammatory condition. The white adipose tissue is an important source of several substances that are linked to inflammatory response and to immunity - the adipokines.

The diets of obese individuals are, usually, rich in saturated and trans fatty acids (FA), and poor in polyunsaturated FA (PUFA), especially, in ω 3 PUFA. These type of dietary habits, by leading to endogenous changes in FA and in lipid metabolism, may, ultimately, lead to changes in the proportions of the different FA in body tissues. The lipid composition of the cell membranes are important determinants in the rheological and physico-chemical properties of the cells, influencing the activity of membrane channels, pumps, transporters and receptors. Thus, it is important in the modulation of cell signalling and in several biological functions.

The lipids of the red blood cell membrane are in constant equilibrium with plasmatic lipids and lipoproteins. While the levels of lipids in plasma are highly influenced by the fasting status, the levels and type of lipids of the erythrocyte membrane reflect the lipid balance of longer periods.

The red blood cell (RBC), as a non-nucleated cell, exhibits a very limited biosynthesis capacity and poor repair mechanisms. Therefore, when exposed to physical and/or chemical stress, during their lifespan, the erythrocyte suffers and accumulates the damage imposed by such stress. In oxidative stress conditions the erythrocyte membrane, may suffer oxidative damage in membrane lipids and proteins. Thus, the erythrocyte is a

good model to study the oxidative damage of lipids and proteins occurring in pro-inflammatory and oxidative conditions and may also provide an important model to study the impact of dietary habits in the lipid and protein composition of the cell membranes.

The objective of this study was to analyse the impact of obesity in the lipid profile, glucose homeostasis and inflammation, as well as the impact of obesity and the associated changes in the RBC membrane composition.

We studied 34 obese children and adolescents [15 (44.1%); mean age of 14.1 years (8-17)] from Hospital S. João and the Children's Hospital Maria Pia. The total group was divided according to the body mass index (BMI) percentile in 17 obese, 8 overweight and 9 controls. Obesity was defined as a BMI higher than the 95th percentile, for age and gender, according to the "2000 Centre for Disease Control and Prevention (CDC) growth charts". Overweight was considered for BMI percentiles equal or higher than 85 and lower than 95; and control subjects BMI were lower than the 85th percentile, adjusted for gender and age. The three groups were matched for age, gender and tanner stage.

It were determined the circulating levels of triglycerides, cholesterol, high density lipoprotein cholesterol, Low density lipoprotein cholesterol, lipoprotein (a), apolipoprotein A, apolipoprotein B, C-reactive protein, glucose, and insulin. A basic hematologic study was also performed. The erythrocyte membrane was studied with the determination of erythrocyte damage markers: membrane bound haemoglobin, proteic carbonylation, lipid peroxidation and band 3 profile - high molecular weight aggregates, monomers and proteolytic fragments. The membrane fatty acid profile was determined.

We found that the obese individuals, presented risk changes in the lipid profile, increased insulin resistance and inflammation, when compared to their lean counterparts. Thus, several changes in cardiovascular disease (CVD) risk markers were observed. No significant changes were found in the erythrogram and in the erythrocyte damage markers.

Concerning the FA membrane profile, the unsaturated FA showed a trend to increase, while saturated FA showed a trend to decrease, with obesity. Despite that, behenic acid (22:0) presented a significantly increase in obese, in comparison with controls. An increasing proportion of 20:0, 18:3n3, 20:3n6 and 22:4n6 were found for overweight and obese individuals, as compared to control. These FA increased with growing obesity, and presented the most significant associations with the studied CVD markers associated with obesity.

Further studies are needed to clarify the associations between membrane FA changes, and CVD risk markers. A larger study, with a higher number of individuals could also clarify some observed trends. Regarding the lipid membrane analysis, some interesting approaches could be made, such as to separate and analyze the different phospholipids and cholesterol ester and their associated FA, and to perform a separate study of inner and outer membrane sheets. Furthermore, optimization of the separation technique and consequent identification of FA would be crucial, as in this study we could not evaluate important FA, e.g. EPA (20:5n3) and GLA (18:3n6), due to technical limitations.

Key words:

Obesity, erythrocyte, inflammation, oxidative stress, dyslipidemia, insulin resistance. metabolic syndrome, fatty acid metabolism.

Contents

Agradecimentos.....	III
Abstract	VII
Figure Contents/Índice de Figuras	XVI
Table Contents/Índice de Tabelas	XIX
Abreviature list.....	XXI
1. Introduction.....	24
1.1. Obesity as a global disease	24
1.2. White adipose tissue – more than a storage depot	25
1.3. Obesity and other diseases	26
2. The Erythrocyte	29
2.1. Introduction.....	29
2.2. Erythrocyte membrane.....	30
2.2.1. Membrane lipids.....	30
2.2.1.1. Composition.....	30
2.2.1.2. Asymmetrical distribution.....	31
2.2.1.3. Exchange with plasma lipoproteins	32
2.2.2. Membrane proteins.....	33
2.2.2.1. Classification	33
2.2.2.2. The Cytoskeleton.....	36
2.2.2.3. Interaction between proteins	37
2.2.2.4. Membrane protein synthesis	38
2.2.3. Lipid-protein interactions.....	39

2.3.	Metabolic pathways	39
2.4.	Cell ageing /damage	41
2.4.1.	Phospholipid externalization	42
2.4.2.	Reduction in sialic acid	42
2.4.3.	Oxidative stress	42
2.4.3.1.	Oxidative damage and anti-oxidant defences.....	44
2.4.4.	Band 3 neo-antigen.....	45
2.4.5.	Removal of the senescent/damaged erythrocytes	48
2.4.6.	Hemoglobin catabolism.....	49
2.5.	Erythrocyte membrane changes in obesity.....	49
3.	Fatty Acids	51
3.1.	Introduction.....	51
3.2.	Chemical structure	52
3.2.1.	Ramification and length of the carbon chain.....	52
3.2.2.	Unsaturations.....	53
3.3.	Nomenclature.....	56
3.4.	Synthesis.....	58
3.4.1.	Synthesis regulation.....	61
3.4.2.	Beyond palmitic acid	62
3.4.3.	Elongation	63
3.4.4.	Desaturation.....	63
3.5.	Fatty acids as energy	65
3.5.1.	Lipolysis	65
3.5.2.	β -Oxidation.....	66
3.5.3.	Peroxisomal oxidation.....	70

3.5.4.	Other oxidation pathways	70
3.5.5.	Ketone bodies	70
3.6.	Linkage to glucidic metabolism	72
3.7.	Dietary fatty acids	73
3.7.1.	Digestion of fatty acids.....	73
3.8.	Types of fatty acids	76
3.8.1.	Free fatty acids	76
3.8.2.	Saturated fatty acids.....	76
3.8.2.1.	Health effects	77
3.8.2.2.	Food sources	77
3.8.2.3.	Dietary needs	77
3.8.3.	Monounsaturated fatty acids	78
3.8.3.1.	Health effects	78
3.8.3.2.	Foods sources	79
3.8.3.3.	Daily needs.....	79
3.8.4.	Polyunsaturated fatty acids	79
3.8.5.	<i>Trans</i> fatty acids	80
3.8.5.1.	Hydrogenation of fatty acids.....	80
3.8.5.2.	Health effects	82
3.8.5.3.	Food Sources.....	83
3.8.5.4.	Dietary needs	84
3.8.6.	Essential fatty acids	84
3.8.6.1.	Biological role.....	85
3.8.7.	Omega 3 fatty acids	85
3.8.7.1.	Health effects	86
3.8.7.2.	Food sources	87

3.8.7.3.	Dietary needs	88
3.8.8.	Omega 6 fatty acids	90
3.8.8.1.	Health effects	90
3.8.8.2.	Food sources	91
3.8.8.3.	Dietary needs	91
3.8.9.	Omega 9 fatty acids	91
3.9.	Fatty acids and inflammation	92
3.9.1.	Omega 6 / Omega 3 ratio	92
3.9.2.	Eicosanoids.....	93
3.9.3.	Omega 3 and omega 6 balance and eicosanoid synthesis	97
4.	Plasmalogens.....	99
4.1.	Synthesis.....	100
4.2.	Distribution in tissues	101
4.3.	Membrane function.....	102
4.4.	Biological function.....	103
4.5.	Analytic procedures for plasmalogens.....	104
5.	Objectives	105
6.	Materials and methods	106
6.1.	Subjects.....	106
6.2.	Procedures and assays.....	106
6.2.1.	Clinical evaluation	106
6.2.2.	Blood samples	107
6.2.2.1.	Basic hematological study.....	107
6.2.3.	Biochemical assays.....	107
6.2.3.1.	Plasma lipids.....	107

6.2.3.2.	Glucose metabolism.....	108
6.2.3.3.	C-Reactive protein	108
6.2.3.4.	Bilirubin.....	108
6.2.4.	Erythrocyte membrane studies.....	108
6.2.4.1.	Preparation of membranes	108
6.2.4.2.	Membrane bound hemoglobin.....	109
6.2.4.3.	Band 3 profile – electrophoretic separation of erythrocyte membrane proteins, immunoblot for Band 3 and densitometric evaluation	109
6.2.4.4.	Membrane lipoperoxidation	110
6.2.4.5.	Membrane protein carbonylation.....	110
6.2.4.6.	Analysis of membrane fatty acids	110
6.2.4.6.1.	Lipid extraction	110
6.2.4.6.2.	Derivatization	111
6.2.4.6.3.	Chromatographic analysis.....	111
6.2.4.6.4.	Chromatogram analysis	112
6.3.	Statistical analysis.....	115
7.	Results and discussion.....	116
7.1.	Lipid profile	117
7.2.	Glucose metabolism and Inflammation	118
7.3.	Erythrogram and markers of erythrocyte damage.....	120
7.4.	Membrane fatty acids	121
7.5.	Linear regression analysis.....	126
7.5.1.	Body mass index	127
7.5.2.	Lipid Profile	130
7.5.3.	Glucose metabolism.....	134
7.5.4.	C-Reactive protein	136

8. Conclusions	138
9. References	141

Figure Contents/Índice de Figuras

Figure 1. Obesity related diseases.	24
Figure 2. Adipocyte secreted products and their impact in body homeostasis.	26
Figure 3. Prevalence of obesity in 15 years old boys and girls from OECD countries.	27
Figure 4. Erythrocytes.	29
Figure 5. Cell membrane structure.	31
Figure 6. Phospholipid movements in the membrane bilayer.....	32
Figure 7. Erythrocyte membrane proteins in SDS page according to Lammeli and Fairbanks. (17)	34
Figure 8. Spatial organization of the Erythrocyte membrane proteins.	35
Figure 9. Cytoskeleton protein interactions.....	36
Figure 10. Spherocytes and eliptocytes in blood smears.	38
Figure 11. Embden-Meyerhof and associated pathways in the erythrocyte.	40
Figure 12. Lipid peroxidation of an unsaturated fatty acid and the peoduction of malondialdehyde and hydroperoxyde.....	43
Figure 13. Hemoglobin oxidation and the promotion of band 3 aggregation and anti-band 3 antibodies binding.	46
Figure 14. Band 3 agreggation opsonization and complement activation.	47
Figure 15. Erythrocyte band 3 protein western-blot.	48
Figure 16. Fatty acid structure.....	51
Figure 17. Types of fatty acid according to the number of unsaturation in the lateral carbon chain.....	53
Figure 18. <i>Cis</i> and <i>trans</i> fatty acid configuration of double bonds in fatty acids.	54

Figure 19. Fatty acids spatial impact of trans and cis double bonds in the carbon lateral chain.	55
Figure 20. Saturated, Monosaturated and Polyunsaturated fatty acids, their structure and melting temperature.	55
Figure 21. Packing of saturated fatty acids and of a mix of saturated and unsaturated fatty acids.....	56
Figure 22. Trivial, systematic and abbreviations used in FA nomenclature.	58
Figure 23. FA synthase structure and the organization of the different catalytic points. ...	59
Figure 24. Fatty acid synthesis reactions performed by fatty acid synthase.....	60
Figure 25. Acetyl-CoA carboxylase regulation	62
Figure 26. Production of oleoyl-CoA from steroyl-CoA through the introduction of a double bon in the carbon chain.	64
Figure 27. Enzymes involved in the synthesis of fatty acids from the 3 omega families. ...	65
Figure 28. Mechanism of entrance of fatty acid acyl-CoA to enter the mitochondrial matrix.	67
Figure 29. Fatty acid β -oxidation cycle.	68
Figure 30. β -Oxidation of unsaturated fatty acid Linoleic acid.....	69
Figure 31. Ketone bodies synthesis.....	71
Figure 32. Transformation of ketone bodies in acetyl CoA.....	71
Figure 33. Link between Fatty acid synthesis, β oxidation and glucose metabolism.	72
Figure 34. Digestion and absorption of triglycerides.	74
Figure 35. Plasmatic lipoprotein and their lipid transport role.....	76
Figure 36. Hydrogenation of <i>cis</i> unsaturated fatty acid with the production of a <i>trans</i> isomer as a side product.	81

Figure 37. Eicosanoids synthesized from arachidonic acid.....	94
Figure 38. Synthesis of PGG ₂ and PGH ₂ from arachidonic acid by cyclooxygenase and peroxidase.	95
Figure 39. Synthesis of 5-HPETE and LTA ₄ from arachidonic acid by 5 lipooxygenase....	95
Figure 40. Plasmalogens structure in comparison to glycerophospholipid and plasmalogen phospholipids.	99
Figure 41. Plasmalogen head groups.	100
Figure 42. Synthetic pathway of plasmalogens.....	101
Figure 43. LnCRP and LnHOMA _{IR} correlation.	119
Figure 44. Behenic acid in the erythrocyte membranes for the studied groups.	122
Figure 45. Odd numbered FA 15:0 and 17:0 vs. BMI percentile groups.....	125
Figure 46. Ratios and enzymes activities of $\Delta 5$, $\Delta 6$ and $\Delta 9$ desaturases, for the studied groups.....	126

Table Contents/Índice de Tabelas

Table 1. List of identified fatty acids and Supelco 37 standard related FAME.	113
Table 2. Groups of fatty acids and ratios.	114
Table 3. Clinical and antropometric characteristics of the studied groups.	116
Table 4. Lipid profile of the studied groups.	117
Table 5. Glucose metabolism and C-reactive protein in the studied groups.....	118
Table 6. Erythrogram and markers of membrane damage.....	121
Table 7. Fatty acid profile observed in the erythrocyte membranes of the studied groups.	123
Table 8. Proportions of different type of fatty acids for the studied groups.	124
Table 9. Linear regression models with markers of adiposity as the dependent variables and fatty acids as independent variables.....	127
Table 10. Linear regression models with adiposity measures as the dependent variables and fatty acids ratios and proportions as the independent variables.....	128
Table 11. Linear regression models with lipid profile variables as the dependent variables and fatty acids as independent variables.....	130
Table 12. Linear regression models with lipid profile variables as the dependent variables and fatty acids ratios and proportions as the independent variables.....	133
Table 13. Linear regression models with glucose homeostasis markers as the dependent variables and fatty acids as independent variables.....	134
Table 14. Linear regression models with glucose homeostasis markers as the dependent variables and fatty acids ratios and proportions as the independent variables.....	135
Table 15. Linear regression models with CRP as the dependent variable and fatty acids as independent variables.....	136

Table 16. Linear regression models with CRP as the dependent variable and fatty acids ratios and proportions as the independent variables.....	136
--	-----

Abreviature list

AA	Arachidonic acid
ACP	Acyl-carrier protein
ALA	α -Linolenic acid
AMP	Adenine monophosphate
Apo	Apolipoprotein
ATP	Adenine triphosphate
BHT	Butylhydroxytoluene
BMI	Body mass index
BMI z-sc	Body mass index z-score
CE	Cholesterol ester
CHD	Coronary heart disease
Chol	Total cholesterol
CLA	Conjugated linoleic acid
CNS	Central nervous system
CoA	Coenzyme A
COX	Cyclooxygenase
CRP	C-reactive protein
CVD	Cardiovascular disease
cAMP	Cyclic adenine monophosphate
CYP	Cytochrome
DGLA	Dihomo- γ -linolenic acid
DHA	Docosahexaenoic
DMA	Dimethylacetal
DNPH	2,4-dinitrophenylhydrazine
DPA	Docosapentaenoic acid
DPG	2,3-diphosphoglycerol
E-EPA	Synthetic eicosapentaenoic ester
EFA	Essential fatty acid
EPA	Eicosapentaenoic
ER	Endoplasmatic reticulum
FA	Fatty acids
FADH ₂	Flavine adenine dinucleotide (reduced)
FAME	Fatty acid methyl-esters
FFA	Free fatty acid
Frag	Band 3 total proteolytic fragments
G3PD	Glyceraldehyde-3-phosphate dehydrogenase
GLA	γ -Linolenic acid
GPx	Glutathione peroxidase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
Hb	Hemoglobin
HDL	High density lipoprotein
HMG	3-hidroxy-3-metilglutaril
HMWAg	Band 3 high molecular weight aggregate
HN	4-hydroxynonenal
HO $\cdot$	Hydroxyl radical
HOMA _{IR}	Homeostasis model assessment insulin resistance
HPx	Hydrogen peroxyde
HIF-1	Hypoxia-inducible factor 1

HSL	Hormone sensitive lipase
Ht	Hematocrit
Ig	Immunoglobulin
IL	Interleukin
Ins	Insulin
IR	Insulin resistance
IUPAC	International Union of Pure and Applied Chemistry
Kcal	Kilocalories
LA	Linoleic acid
LDL	Low density lipoprotein
LOX	Lipoxygenase
LP	Lipid peroxidation
Lp(a)	Lipoprotein (a)
LPL	Lipoprotein lipase
LR	Lipid rafts microdomains
LT	Leucotriens
MBH	Membrane bound hemoglobin
MCH	Mean cellular hemoglobin
MCHC	Mean cellular hemoglobin concentration
MCP-1	Monocyte chemoattractant protein 1
MCV	Mean cellular volume
MDA	Malonildialdehyde
MetHb	Methemoglobin
Mon	Band 3 monomer
MS	Metabolic syndrome
MUFA	Mono unsaturated fatty acid
NAD	Nicotinamide adenine dinucleotide (oxidized)
NADH	Nicotinamide adenine dinucleotide (reduced)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NO	Nitric oxide
oxLDL	Oxidized LDL particles
PA	Phosphatidic acid
PAI	Plasminogen activator inhibitor
PBS	Phosphate buffered solutio
PC	Phosphatidylcholine
PCb	Protein carbonylation
PE	Phosphatidylethanolamine
PG	Prostaglandin
PGI	Prostaciclins
PI	Phosphatidylinositol
PIP2	Phosphatidylinositol-4,5-bisphosphate
PIP	Phosphatidylinositol-monophosphate
PL	Phospholipids
PLA2	Phospholipase A2
PlsC	Glycerophosphoryl-choline plasmalogens
PlsE	Glycerophosphoryl-ethanolamine plasmalogens
PRX2	Peroxiredoxin 2
PS	Phosphatidylserine
PUFA	Poly unsaturated fatty acid
RBC	Red blood cell
RBP	Retinol binding protein
RDW	Red cell distribution width
RES	Reticulo-endothelial system
ROS	Reactive oxygen species
SFA	Saturated fatty acid

SDS	Sodium dodecylsulphate
SM	Sphingomyelin
SOD	Superoxide dismutase
T2DM	Type 2 diabetes mellitus
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
TG	Triglycerides
TNF- α	Tumor necrosis factor- α
TX	Thromboxanes
UI	Unsaturation index
VEGF	Vascular endothelial growth factor
VLDL	Very low density lipoprotein
VLPufa	Very long chain poly unsaturated fatty acid
W/H	Waist-to-height
WAT	White adipose tissue

1. Introduction

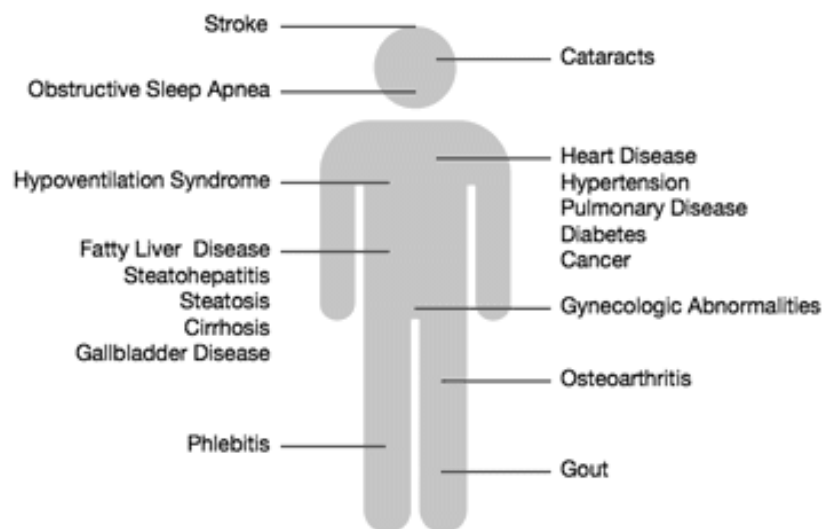
1.1. Obesity as a global disease

Obesity is increasing all over the world and is present in both developed and underdeveloped countries, in the recent years. (2)

Modern lifestyle seems to be the main responsible for this “pandemia”. Sedentarism, lack of physical exercise, carbon hydrate and fat rich diets, snacking and long hours of television watching, are some of the many features of an unhealthy routine that has been perpetuated in many families. Globally, with this lifestyle, there is an increase in the energy intake and reduced expenditure, leading to an increase in body weight and in the percentage of body fat. (3-4)

Adolescent and childhood obesity have also reached a high prevalence in many countries. A special attention should be given to this group of the population, as obesity in pediatric and adolescent ages have been related with the development of many diseases in the future, as adults (e.g. diabetes type 2, metabolic syndrome, etc.). In fact, many studies point to the fact that atherosclerosis, is a chronic disease that begins early in life, and that obesity is an important risk factor for the development of future cardiovascular disease (CVD). Figure 1 presents some of the diseases related with obesity. (3-4)

Figure 1. Obesity related diseases.



Adapted from internet. (5)

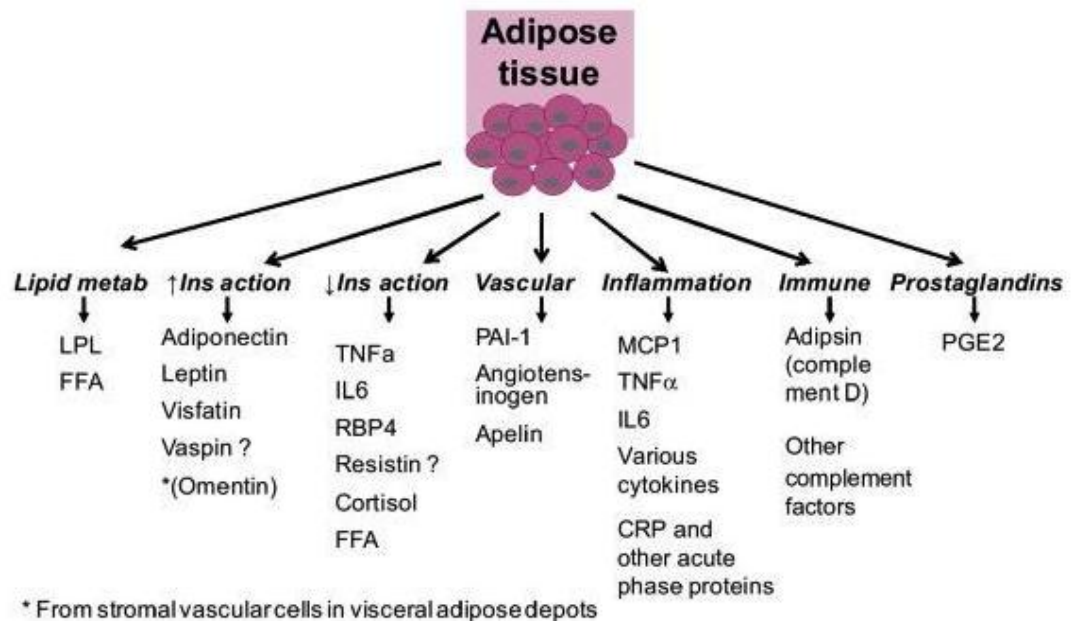
1.2. White adipose tissue – more than a storage depot

The weight gain that characterizes obesity is mainly due to an increase in the mass of white adipose tissue (WAT) of the body. Until some years ago, this tissue was simply considered as a place of storage of lipid energetic reserves for the organism, but, recently, its function and complexity as an active endocrine organ has been largely explored. (3-4)

The secretion of tumor necrosis factor- α (TNF- α) and leptin by the WAT have been described and a surprising number of other active substances produced by this tissue have been described. For some of them, like leptin and adiponectin, the WAT is, in fact, the major source, especially in obese individuals. (6-7)

The compounds produced by the WAT were named as adipokines. The majority of these molecules - cytokines, chemokines, acute phase proteins – play a role in the inflammatory response. In the WAT, the adipokines are secreted by adipocytes, pre-adipocytes, infiltrated macrophages and cells of the vascular stromal fraction. The production of adipokines, usually, increases with increasing adiposity, except adiponectin, which is an anti-inflammatory adipokine. (8) Thus, the majority of the products secreted by the WAT produce are pro-inflammatory. When their production increases, the rise is observed not only within the WAT itself, but also in the circulation. In obesity, inflammation is not restricted to the WAT. Rather, there is an intense dialogue between the WAT and other tissues and organs, leading to a state of chronic mild inflammation. For example, the interleukin (IL)-6 secreted by the WAT will induce, through the venous portal system, the secretion by the liver of C-reactive protein (CRP), a sensitive inflammatory mediator. (8) Figure 2 shows the relation between some adipokines and their impact in body homeostasis. (3-4)

Figure 2. Adipocyte secreted products and their impact in body homeostasis.



CRP, C reactive protein; FFA, free fatty acid; IL6, interleukin 6; Ins, insulin; LPL, lipoprotein lipase; MCP1, monocyte chemoattractant protein; PAI-1, plasminogen activator inhibitor-1; PGE2, prostaglandin E2; RBP4, retinol binding protein; TNF- α , tumour necrosis factor. Adapted from internet. (9)

Inflammatory states, such as obesity, are known as oxidative stress conditions. The development of oxidative stress results from inflammatory cell activation, with release of reactive oxygen species, and to the lowering in the anti-oxidant defenses. (10-12)

Thus, the WAT, instead of being just a depot tissue, is an active secretory organ that is in a close cross-talk with other organs and tissues of the body. The WAT is one of the few body tissues presenting almost unlimited growing potential. Therefore, it is easy to imagine the impact that an active secretory organ, which can increase many times its size, may have in body's physiology. (3-4)

1.3. Obesity and other diseases

Obesity plays a very important role in the etiology of some diseases, like type 2 diabetes mellitus (T2DM), CVD and metabolic syndrome (MS). (3-4)

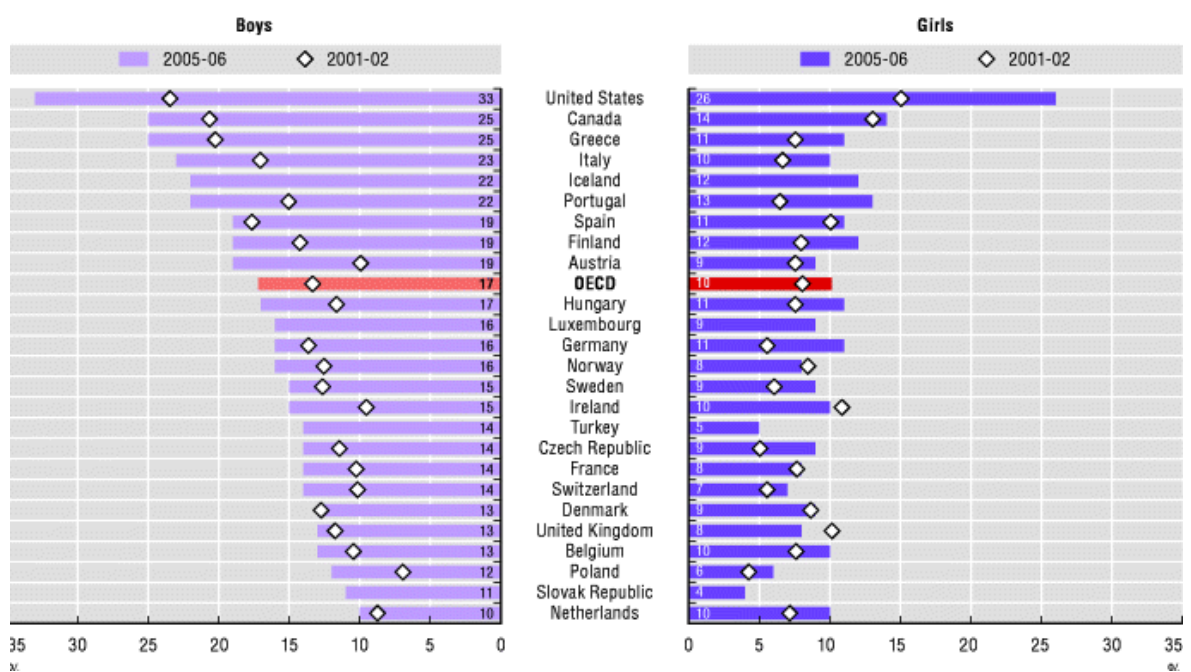
The MS is, usually, defined as a cluster of risk factors for CVD, including dislipidemia, hypertension, insulin resistance (IR), impaired glucose metabolism and obesity itself.

Some of the mechanisms underlying the association of obesity with T2DM, CVD and MS are still unknown and are subject of intense investigations, however, inflammatory and oxidative stress mechanisms are accepted as underlying mechanisms. In fact, inflammation is known to play a vital role in the initiation and progression of the atherosclerotic vascular disease, in IR, dislipidemia, and other diseases. (13-14)

Atherosclerosis is a chronic disease that begins early in life, and obesity is an important risk factor for its development. In fact, atherosclerotic changes have been described in the vessels of increasingly younger subjects. Also, childhood and adolescent's obesity seems to increase the risk of developing T2DM in adulthood, and the presence of MS features are already observed at low ages. (15-16)

This phenomena of childhood obesity is particularly important in our country, as a high prevalence of overweight/obesity (over 30%) has been reported for Portuguese children, as compared to other European countries. (15-16) Figure 3 presents the results of a study involving 15 year old boys and girls from different countries, members of the OECD (Organization for Economic Co-operation and Development). Portugal has a high prevalence of childhood obesity, in comparison with the other countries. This high prevalence is shared with other southern European countries, as Greece, Italy and Spain, in contrast with lower prevalence in the northern countries. There is a trend for increasing obesity from the first studied period to the second, for almost all countries, although for some countries the prevalence of obesity in girls appears to be diminishing. (3-4)

Figure 3. Prevalence of obesity in 15 years old boys and girls from OECD countries.



OECD (Organization for Economic Co-operation and Development). Adapted from (2).

Although obesity is known to be linked with many diseases, the cross-talk mechanisms between them are very important issues for public health nowadays and will probably be even more in a close future, especially as the population is becoming older and fatter. To better understand these closed net of inter-relations, it is necessary, in a first approach, to consider each disease mediator individually and, later, to construct the complex map of biological relations between obesity and other diseases.

2. The Erythrocyte

2.1. Introduction

The erythrocyte is the blood cell responsible for the oxygen transport from the lungs to the peripheral tissues and to mediate carbon dioxide transport from the tissues back to lungs, where it will be expelled. (17)

The red blood cell (RBC) presents with a biconcave disk shape (discocyte) (Figure 4), and this particular shape is of great importance for the cell, as it confers to the erythrocyte an outstanding surface area, flexibility and deformability, necessary to deliver oxygen to the whole body. As it moves through the vessel network of the body, the RBC will have to circulate through vessels of reduced diameter, such as the capillary vessels, with a lumen smaller than its own size. Thus, the discocyte shape helps turning this task possible. (18)

Figure 4.
Erythrocytes.



As a non-nucleated cell, the erythrocyte exhibits a very limited biosynthesis capacity and poor repair mechanisms. Therefore, when exposed to physical and/or chemical stress, during its lifespan, the RBC suffers and accumulates the damage imposed by such stress. (19-20) The rise in inflammatory cells and in their activation products, as oxygen reactive species and proteases, are some of the important promoters of oxidative and proteolytic damage in erythrocytes. (21-22) Thus, the role of delivering oxygen, together with the poor RBC biosynthetic and repair machinery, turns this cell particularly vulnerable to oxidative modifications. (17)

A cumulative oxidative damage of the RBC may occur with cell aging or when under oxidative stress conditions. The oxidative damages will affect the structure of the membrane, cell flexibility and lead to changes in cell shape, from a discocyte to a spherocyte, due to membrane vesiculation. The spherocyte, presenting a decreased capacity of deformability, will be sequestered within the splenic sinusoids and removed from circulation. (23)

Due to its simple organization, without organelles and nucleus, the RBC represents a very good model to study oxidative stress conditions, as inflammation and obesity.

2.2. Erythrocyte membrane

The erythrocyte membrane is, in its basic structure and functionality, similar to the other cell membranes of the human body. However, we can find particular points in the RBC membranes. (17)

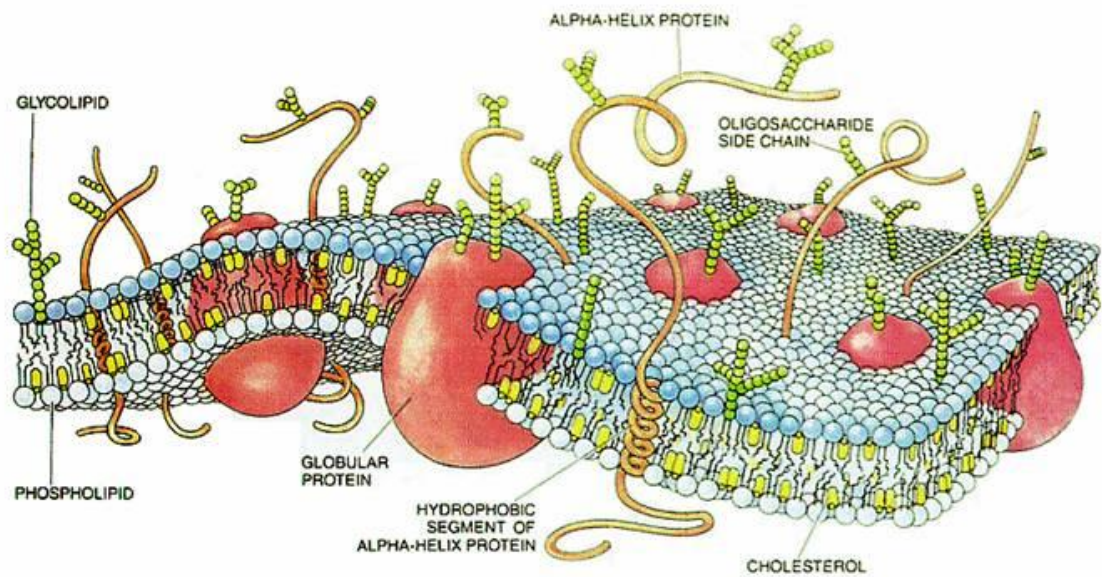
The erythrocyte membrane has an asymmetrical lipid bilayer (32.5-44% phospholipids and 11% cholesterol w/w), proteins (49% w/w), and carbohydrates (7% w/w). The lipid fraction includes about 60% of phospholipids (PL), 30% of neutral lipids (mainly free cholesterol) and 10% of glycolipids. As in other biological membranes, its double layer has a hydrophobic core, formed by the hydrophobic tails of the fatty acids (FA) from PL, and two hydrophilic sides, one facing the cytoplasm (cytoplasmatic/inner leaflet) and the other facing plasma (plasmatic/outer leaflet). (17)

2.2.1. Membrane lipids

2.2.1.1. Composition

The main PLs of the membrane are phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin (SM) and phosphatidylserine (PS). Other minor PLs are also present, such as phosphatidylinositol (PI), PI-monophosphate (PIP), PI-4,5-bisphosphate (PIP₂), phosphatidic acid (PA), lysophosphatidylcholine (lyso-PC) and lysophosphatidylethanolamine (lyso-PE). Glycolipids and unesterified cholesterol are intercalated between membrane phospholipids. Cholesterol is equally distributed between the two layers, while glycolipids are localized in the outer leaflet. (Figure 5) These glycolipids, and glycoproteins, are important as signaling RBC molecules (e.g. blood group antigens). (24-25)

Figure 5. Cell membrane structure.



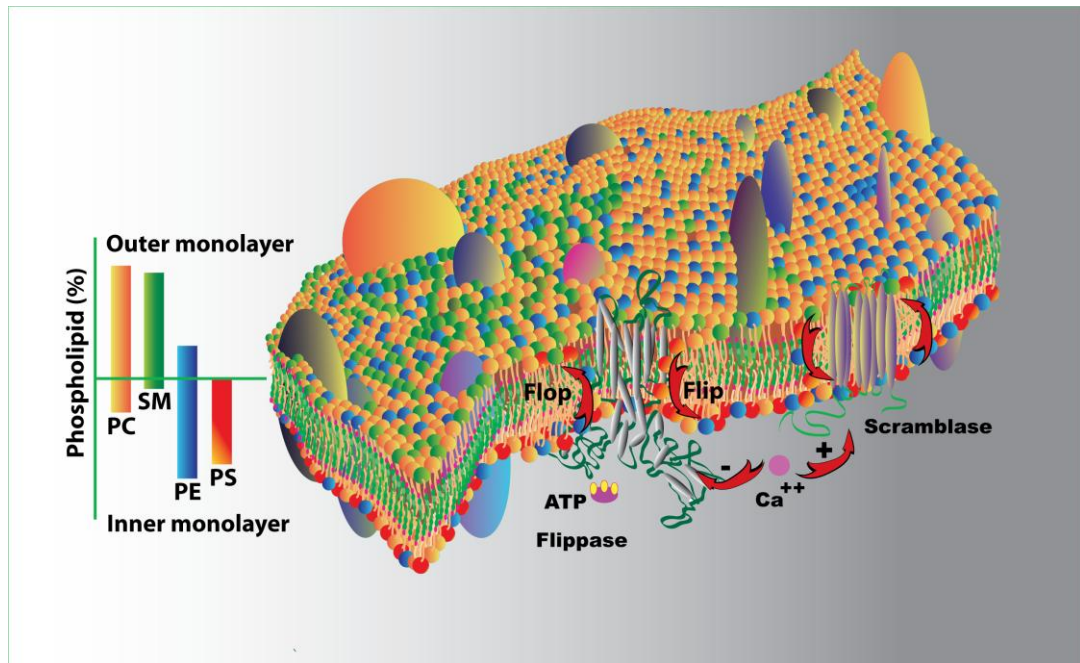
Adapted from internet. (9)

2.2.1.2. Asymmetrical distribution

The erythrocyte membrane is an asymmetrical structure. This asymmetry is present in the distribution of PL between the two layers of the membrane; PS and PE are mainly present in the inner side of the membrane, while PC and SM are predominantly located in the outer leaflet of the bilayer (Figure 6). (24-25)

The asymmetry of the membrane is maintained through the movement of PL from one layer to the other. The movement that allows PL to change position between the two layers is called “flip-flop”. Although phospholipids diffuse rapidly in the same side of the membrane, their polar head groups cannot easily pass through the hydrophobic center of the bilayer, limiting their diffusion between leaflets (Figure 6). (17)

Figure 6. Phospholipid movements in the membrane bilayer.



PC, phosphatidylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin; PS, phosphatidylserine. Adapted from internet. (26)

Some membrane proteins are involved in this process, such as the ATP dependent aminophospholipid translocases or “flippases” (from the outer to the inner layer), the phospholipid translocases or “floppases” (from the inner to the outer layer) and calcium dependent “scramblases” (promote PL movements in both ways). The exteriorization of PS and PE to the outer leaflet of the PL bilayer of the membrane has been associated to senescence of the erythrocyte. In other biological cells, the externalization of PS is, in fact, a marker for apoptosis. In the RBC, the presence of this PL in the outer leaflet leads to increasing adhesion of these cells to the vascular walls and also favors coagulation. (24-25)

2.2.1.3. Exchange with plasma lipoproteins

Due to its poor synthetic capacity, the RBC cannot synthesize lipids; therefore, the cell is dependent on the exchange with plasma lipids, to replace damaged lipids. Cholesterol is easily traded, while external PL, as PC and SM, are exchanged in a much slower rate. The PLs from the internal layer are not traded at all. After the exchange with plasma, the fatty acids are acylated in the membrane through an ATP-dependent process, and combined

with lysophosphatides (precursors of phospholipids), to remake the native PL, renewing the damaged or lost fatty acid side-chains. (24-25)

Measurement of PL in plasma is highly influenced by fasting status. In contrast, PL of the erythrocyte membrane reflect the lipid balance of longer periods. (27)

2.2.2. Membrane proteins

2.2.2.1. Classification

More than 50 different erythrocyte membrane proteins have been already described; however, the function of many of those proteins is still unknown. (17)

The erythrocyte membrane proteins are grouped according to their position in the membrane bilayer or according to their role in the membrane structure:

According to its position in the membrane protein, they are classified, as:

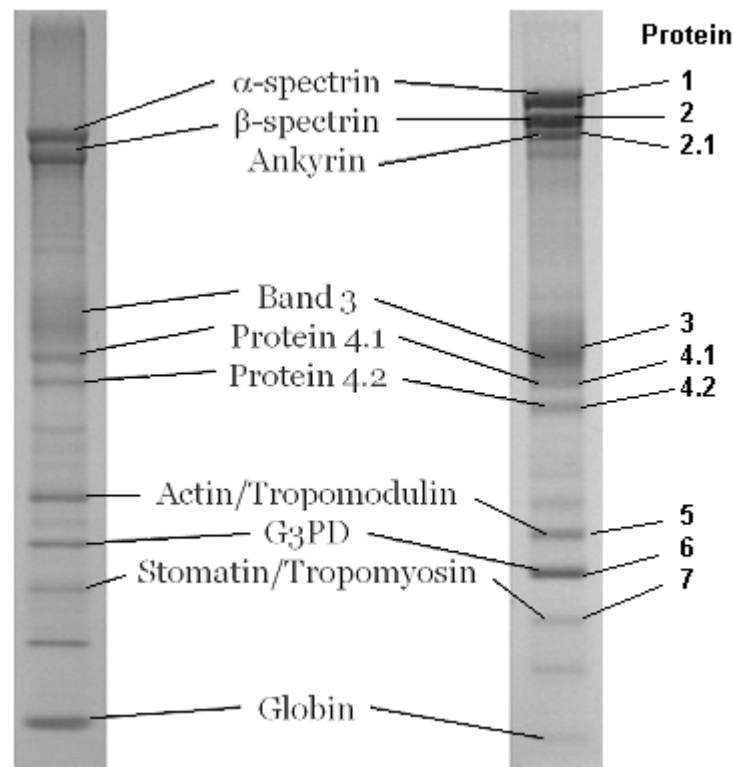
- Integral Proteins: these proteins cross the hydrophobic core of the membrane going from one layer to the other (e.g. Band 3 and glycophorins).
- Peripheral proteins: these proteins are localized in the outer or in the inner side of the membrane bilayer. The inner leaflet proteins are, usually, part of or related to the cytoskeleton. The outer leaflet proteins are usually erythrocyte determinant antigens. (17)

According to their role in the membrane structure, the protein are classified, as:

- Cytoskeletal proteins – as spectrin (α and β chains), protein 4.1, and actin (28)
- Integral/transmembrane proteins – as Band 3 and glycophorins (29)
- Anchoring/linker proteins – as ankyrin and protein 4.2 that mediate the attachment of cytoskeletal proteins to integral proteins. (30)

The main proteins of the RBC membranes are known either by their name, normally based on their function or structure, or according to their mobility in a polyacrilamide gel electrophoresis in the presence of sodium dodecylsulphate (SDS-PAGE) (Figure 7).

Figure 7. Erythrocyte membrane proteins in SDS page according to Lammeli and Fairbanks. (17)



G3PD, glyceraldehyde-3-phosphate dehydrogenase

The more representative RBC membrane proteins and their function, are listed: (17)

Protein 1 and 2 – α and β chains of spectrin, respectively. Spectrin is the major protein of the cytoskeleton.

Protein 2.1 – Ankyrin – cytoskeleton protein that is linked to protein 4.2 and anchors Band 3.

Band 3 – A transmembrane protein, also known as the anion channel, due to its important role in the exchange of HCO_3^- and Cl^- ions through the membrane.

Protein 4.1 – A cytoskeleton protein, important in membrane stabilization through the anchorage of glycophorin C.

Protein 4.2 – A cytoskeleton protein that is linked to Band 3 and to ankyrin.

Protein 4.5 – A transmembrane protein, important as glucose transporter through the membrane, which is independent of energy.

Band 5 – Actine - cytoskeleton protein, linked to glycophorin C, together with protein 4.1, spectrine, tropiomiosine and tropomoduline.

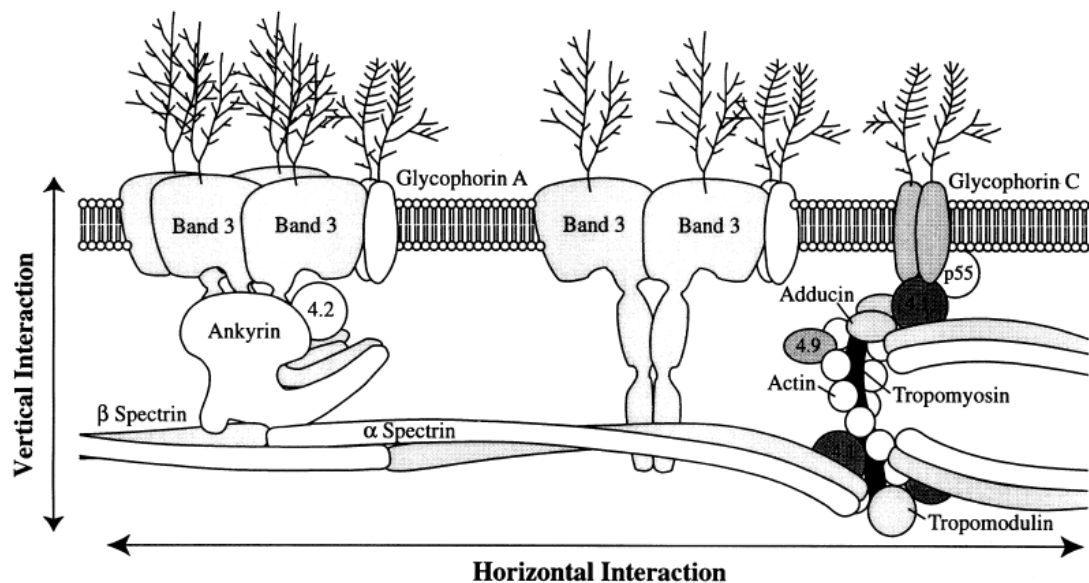
Protein 6 - the enzyme glyceraldehyde-3-phosphate dehydrogenase (G3PD).

Protein 7 – Estomatine - important for the stability of RBC shape; its deficiency causes an hemolytic anemia, called Hereditary Stomacytosis (the erythrocytes acquire a mouth-like shape).

PAS 1 and 2 - glycophorines (sialoglycoproteins). There are five glycophorins, named from A to E; glycophorin C is the most important glycophorin as, besides its role in linking the lipid bilayer to the cytoskeleton, it is responsible for the presentation of erythrocyte antigens determining important blood groups; the other glycophorins have receptor functions.

Figure 8 presents the spatial organization of the major RBC membrane proteins.

Figure 8. Spatial organization of the Erythrocyte membrane proteins.



Adapted from Tse *et al.* (31)

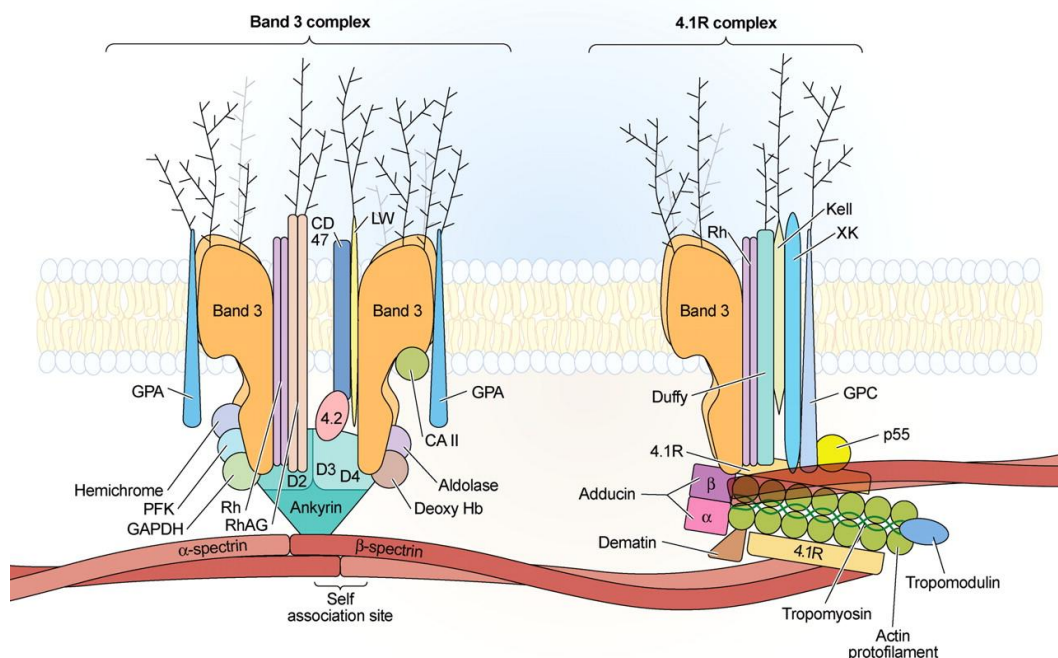
2.2.2.2. The Cytoskeleton

The cytoskeleton is a two-dimensional group of proteins present in the cytoplasmic leaflet of the membrane that are responsible, through its interaction between each other and with the transmembrane proteins, to keep the shape and flexibility of the cell. The major cytoskeleton proteins are spectrin (α - and β -chains), ankyrin, protein 4.1, protein 4.2 (or 4.1R), and actin. (25, 32-33)

Spectrin is the main protein of the cytoskeleton, representing about 35% of its proteins. It is a heterodimer of α spectrin and β spectrin chains. The α and β spectrin chains intertwine in an antiparallel manner to form 100-nm-long heterodimers that self-associate with other $\alpha\beta$ -spectrin heterodimers to form $(\alpha\beta)_2$ heterotetramers, the functional spectrin subunit in the erythrocyte. (28)

The spectrin can link with other membrane proteins through two different sites (Figure 9):

Figure 9. Cytoskeleton protein interactions.



GP, Glycophorin. Adapted from An *et al.* (33)

- Glycophorin C: in this linkage protein 4.1 interacts with the N-terminal of β spectrin and with actine filaments. Dematin, tropomyosin, β adducin and tropomodulin, that are less representative proteins, are also linked to actin and seem to contribute to the stabilization of the protein complex. Outside of these complex protein 4.1 binds to glycophorin C and p55 (18, 24-25, 32-36)
- Band 3: this linkage is centered by a band-3 tetramer (although Band 3 can also exist as a dimer) linked to ankyrin and protein 4.2. The cytoplasmic domain of Band 3 anchors ankyrin (18, 24-25, 32-36) (that binds to the C-terminal region of the β -spectrin chain and to protein 4.2 (37); the latter also binds to Band 3 (38) and it is believed that this linkage is essential for the stability of the membrane. Glycophorin A is also involved in this complex. It is also present the Rh complex, formed by Rh polypeptides, Rh-associated glycoprotein (RhAG), CD47, glycophorin B and the Landsteiner-Wiener glycoprotein. The protein CD47 interacts with protein 4.2 and ankyrin to form a macro complex with band 3. (17)

2.2.2.3. Interaction between proteins

There are two kinds of interactions between the membrane proteins (Figure 8 and 9):

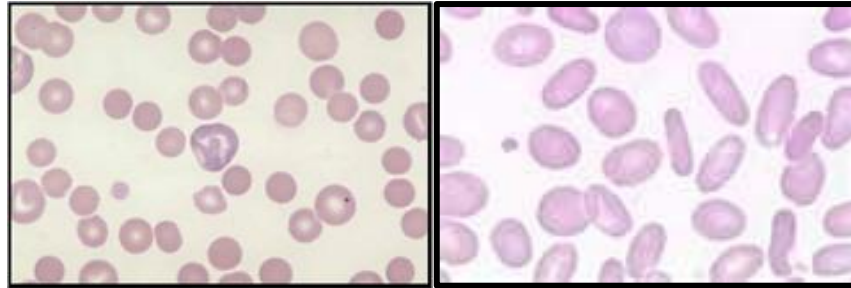
- Vertical: between the cytoskeleton and the transmembrane proteins.
- Horizontal: between the proteins of the cytoskeleton.

Changes in these interactions will lead to the distortion of RBC shape. Changes in the vertical protein interactions will trigger destabilization of the membrane structure, favoring the loss of small membrane vesicles (membrane vesiculation), which is responsible for a cell shape change, from a discocyte to a spherocyte. These spherocytic RBC are less flexible, due to a more rigid membrane, and will be more easily removed from circulation and destroyed. These changes in vertical protein interaction, by causing destabilization of membrane structure, lead to the development of hemolytic anemias, such as Hereditary Spherocytosis. The causes for Hereditary Spherocytosis are deficiencies in some proteins, namely Band 3, spectrin, ankyrin and protein 4.2. (17)

Changes in the horizontal protein interactions also lead to an altered membrane structure leading a cell shape change, from discocyte to an eliptocyte, a more elongated cell. Deficiencies in spectrin or protein 4.1 cause Hereditary Elipocytosis, also an hemolytic anemia, which is, usually, milder than Hereditary Spherocytosis. (17)

Figure 10 presents two slides presenting spherocytes and eliptyocytes.

Figure 10. Spherocytes and eliptyocytes in blood smears.



Adapted from internet. (39)

2.2.2.4. Membrane protein synthesis

To maintain the cell shape, the RBC must have a fine protein network, which is achieved by the correct linkage between several membrane proteins. The process of the synthesis of the erythrocyte membrane proteins is still poorly understood; indeed, along erythropoiesis, some proteins seem to be produced in excess, leading to the presence of limiting points in the cytoskeleton building process. One of these unclarified point is the fact that spectrin α is produced 4 times more than spectrin β , in the erythroid precursors cells. In these cells, the synthesis of spectrin, ankyrin, protein 4.1 and glycophorins, begin before starting the synthesis of Band 3. However, until Band 3 provides a proper place in the membrane to the anchorage of spectrin, ankyrin and other proteins of the cytoskeleton, only a small amount of spectrin and ankyrin is incorporated in the membrane. (17)

The process seems to begin by the binding of spectrin heterodimer to ankyrin; afterwards, ankyrin-linked to spectrin, binds to Band 3; protein 4.2 in one of the last to be produced and appears only in the latest stage of erythroblast differentiation. Its linkage stabilizes the vertical interaction between Band 3 and ankyrin. (40-42)

In the late erythroblasts, when the synthesis of spectrin, ankyrin and Band 3 is already more reduced, the synthesis of protein 4.1 is increased, being synthetized in excess at this phase. The proteins that are synthesized in excess, are rapidly degraded. (40-42)

2.2.3. Lipid-protein interactions

Membrane lipids and proteins interact with each other inside the bilayer. These interactions will lead to a non-random distribution of both proteins and lipids inside the membrane, causing an even more asymmetric structure of the RBC membrane. The transmembrane proteins, like Band 3, maintain its channels and pumps stable through a close relation with the membrane lipids. Spectrin and protein 4.1, both peripheral proteins from the cytoskeleton, bind to PL from the cytoplasmatic leaflet of the membrane, mainly, to the anionic PS and PI. From this interaction between those PLs and proteins results a particular distribution of the lipids in the membrane that is not random. It appears that the spectrin binding to PS increases membrane mechanical stability, while the interaction between PI and protein 4.1 regulates its interaction with integral proteins as band 3 and glycophorin C. (29-30)

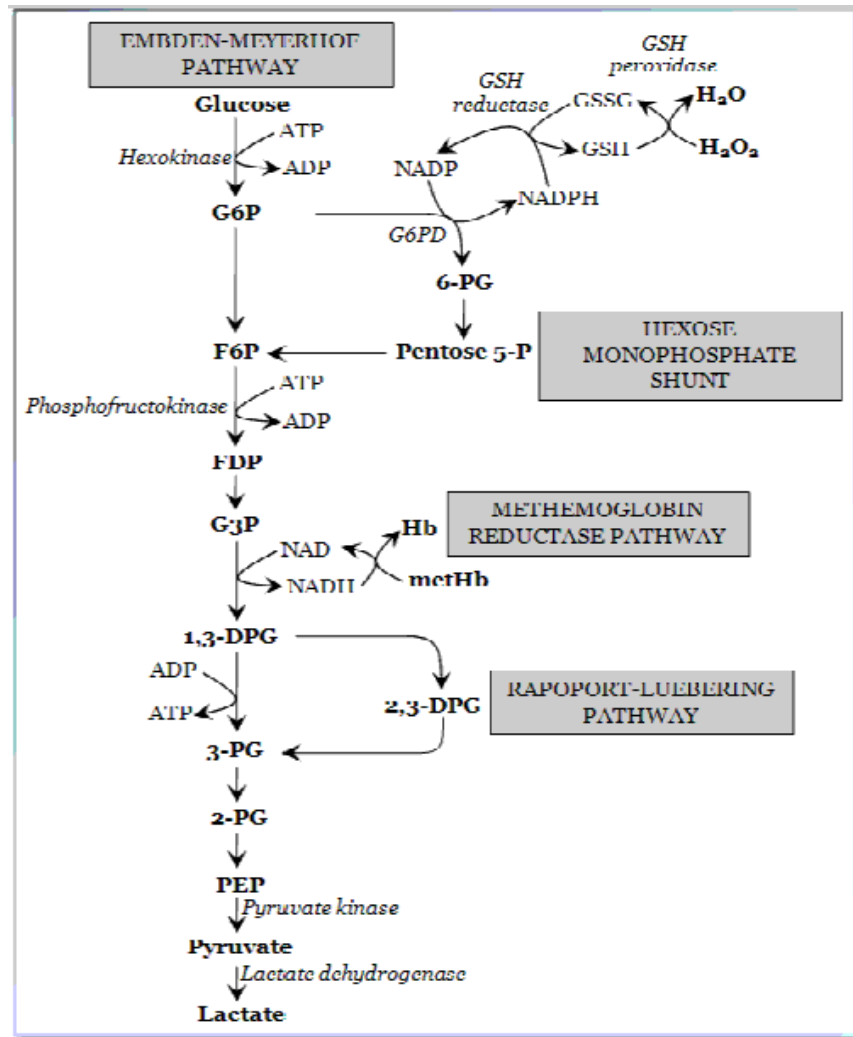
The presence of “lipid rafts” (LR) inside the membrane has been described. These LR are detergent resistant microdomains, rich in cholesterol, sphingolipids and in PL with largely saturated fatty acid residues. In these microdomains, there is also a higher density in some proteins, such as glycosylphosphatidylinositol-linked proteins. The precise functions of these specific domains are still poorly clarified. (29-30)

2.3. Metabolic pathways

The erythrocyte has low needs of energy, though it is still necessary for some cellular processes, e.g. active transport through the membrane, maintenance of membrane lipid asymmetry, and cyclic adenine monophosphate (cAMP) dependent kinases. (17)

The majority of the erythrocyte energy (around 90%) is obtained from glucose, by anaerobic glycolysis - the Embden-Meyerhof pathway (Figure). Lactate, adenine triphosphate (ATP) and reduced nicotinamide adenine dinucleotide (NADH), result from this pathway. (23, 43-44)

Figure 11. Embden-Meyerhof and associated pathways in the erythrocyte.



Adapted from Rocha, S (17).

The energy provided from ATP is necessary to keep the cellular homeostasis, namely, the maintenance of hemoglobin (Hb) in its reduced active form, membrane integrity, replacement of membrane lipids, active protein pumps to keep the osmotic balance and allow the entrance of important substrates. (17)

Hexokinase, phosphofructokinase and pyruvate kinase are enzymes that regulate the glycolytic rate. The remaining 10 % of glucose is used in the hexose monophosphate (or pentose-phosphate) shunt that is fundamental for the production of reduced nicotinamide adenine dinucleotide phosphate (NADPH) in the cell (Figure 11). This molecule will help to maintain the reducing intracellular state, preventing oxidative damages. (23, 43-44)

The affinity of Hb to oxygen is controlled through the levels of 2,3-diphosphoglycerol (DPG), which is produced by the Rapoport-Luebering shunt (Figure 11). (23, 43-44)

The nature of RBC and the linkage of oxygen to Hb lead to a potential pro-oxidant state. The iron in the Hb molecule is in the ferrous (2+) form, but it can be easily oxidized to the ferric (3+) form, with production of methemoglobin (metHb) and superoxide anion (O_2^-). MetHb is not functional and has to be reduced back to its reduced form. The reduction of MetHb back to Hb is mediated by the metHb reductase enzyme system, which requires NADH and/or NADPH, produced by glycolysis and cytochrome b5 as electron carriers (Figure 11). (23, 43-44)

Some enzymes present a balance between their membrane bound and cytosolic forms. Aldolase, glyceraldehyde-3-phosphate dehydrogenase (G3PD) and phosphoglycerate kinase, linked to the production of ATP, are thought to form a membrane-bound enzyme complex, in which G3PD (band 6 in membrane protein SDS PAGE) is in higher amount. In fact, about 90% of G3PD is bound to the cytoplasmic domain of Band 3, in its inactive form. (45-48)

ATP consuming proteins, as adenylyl cyclase, protein kinases and ATPases are also bound to the membrane. (17)

2.4. Cell ageing /damage

The RBC has a limited capacity to face external damage. As an anucleated cell, without organelles, it is not able to synthesize or repair the damages. Therefore, it will accumulate those damages suffered during its life span (about 120 days). Older RBC are smaller, denser and more rigid. Their structural and enzymatic changes with cell aging, have been studied during the last years. The senescence/damage of the RBC appears to be more closely related to changes occurring in the cytoplasmic membrane than with the enzymatic turnover. (49-51)

2.4.1. Phospholipid externalization

As the RBC ages, there is an externalization of PS and PE from the inner to the outer leaflet of the membrane; however, PS appears to have a more important role in RBC senescence. This particular PL is known to be a marker of apoptosis in other biological cells. (17)

There are still some missing points to understand the role of PS externalization in RBC senescence. It has been proposed that this externalization of PS leads to complement activation marking RBC for binding to macrophages, and, therefore, for its removal; the PS externalization is increased in some diseases, such as sickle cell anemia, β -thalassemia and diabetes, and is normal in hemolytic anemias, such as hereditary spherocytosis; in vitro experiments with pro-oxidants did not increase the levels of PS externalization (52-53); the PS externalization increases the adhesion of RBC to endothelial cells and it also seems to favor the coagulation process. (25, 28)

2.4.2. Reduction in sialic acid

Sialic acid, the principal responsible for the erythrocyte negative charge, is linked to RBC senescence undergoing a decrease with RBC age, and may play a role in its removal. (17)

The reduction in sialic acid, linked to glycophorin, leads to a decrease in the RBC negative membrane charge (together with the externalization of certain PL), favoring RBC aggregation and adhesion to endothelial cells. (54-59)

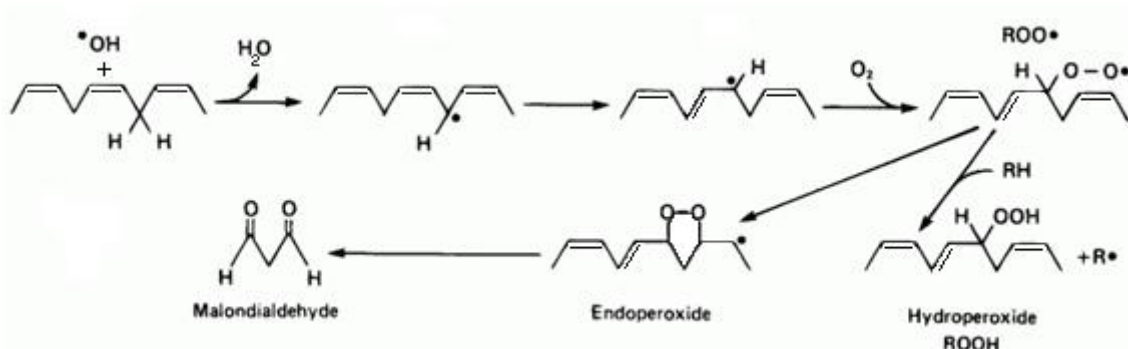
2.4.3. Oxidative stress

When the Hb is deoxygenated, the oxygen binds to iron from the heme group, sharing one electron with oxygen, forming, thus, a superoxide transitory form. When the hemoglobin deoxygenation takes places, the oxygen is released and the electron returns to its initial position. When, along the dissociation of oxygen from the hemoglobin, the shared electron is taken by the oxygen, a superoxide radical and methHB are formed. In this case, the superoxide radical undergoes spontaneous dismutation and, in the presence of

superoxide dismutase originates hydrogen peroxide (HPx) and oxygen. When the superoxide is not promptly converted, it can react with HPx, in the presence of a transition metal (as iron – Fenton reaction), and originate hydroxyl radical ($\text{HO}^\bullet$), the most reactive radical particle in biological systems. The metHb that is formed will be reduced back to its functional reduced form, by the metHb reductases. When these mechanisms fail, the metHb will accumulate within the cell in the form of hemicromes that will affect the RBC structure. (56, 60-62)

The hydroxyl radical has an enormous potential of reacting with biomacromolecules and to induce oxidative damage. It may react with the membrane unsaturated FA of the PL through the removal of one hydrogen from the double bond, with formation of a hydroperoxide. This process is called lipid peroxidation (LP) (Figure 11) The LP secondary products, such as malondialdehyde (MDA) and 4-hydroxynonenal (HN), can attack membrane skeletal proteins and have other deleterious actions. (63) The hydroxyl radical also attacks proteins directly, besides through the action of those secondary products. The damage in proteins, usually, involves the addition of a carboxyl group – protein carbonylation – and the formation of protein centered alkylperoxyl radicals. (56, 60-62)

Figure 12. Lipid peroxidation of an unsaturated fatty acid and the production of malondialdehyde and hydroperoxide.



Adapted from internet. (64)

There are also oxidants that can cause Hb oxidation, namely, nitric oxide (NO), HPx and hydroxyl radical, that may be produced by endothelium cells and inflammatory cells (neutrophils, macrophages/monocytes). Indeed, leukocyte activation induced-oxygen metabolites, by diffusing across the RBC membrane, may also trigger an oxidant stress

within the cell and the consequent hemoglobin oxidation if the antioxidant mechanisms of the cell fail. (21, 65) Oxidants may also come from food and drugs. (56, 60-62) Nitric oxide is an important molecule used as a signaling messenger by endothelium cells and macrophages, which is synthesized through NO-synthase. It is also an oxidant agent that will increase its oxidant activity when forms peroxynitrites by reacting with superoxide radical. (56, 60-62)

2.4.3.1. Oxidative damage and anti-oxidant defences

The RBC contains enzymatic and non-enzymatic defenses against oxidative damage. The most important antioxidant enzymes are the superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), peroxiredoxin 2 (PRX2) and glutathione reductase. The most important non-enzymatic antioxidants include reduced glutathione (GSH) (the most common reducing agent in the erythrocyte). Superoxide dismutase is responsible for the reduction of superoxide radical to hydrogen peroxide that should be eliminated by catalase, GPx and PRX2. Catalase reduces HPx into water and molecular oxygen; PRX2, the third most abundant protein in the RBC, is thiol dependent and based in the thioredoxin/thioredoxin reductase/NADPH reducing system. (66-68) GPx reduces HPx into water by oxidizing GSH into GSSG (oxidized glutathione). The GPx and PRX2 are also capable of scavenging hydroxyl radicals and hydroperoxides resulting from LP. Oxidized glutathione is transformed back into its reduced form by GR, using NADPH as a cofactor. . (44, 60, 66-69)

Thus, NADPH is important for the activity of PRX2 and GPx, by regenerating GSH. In the RBC, NADPH is produced only through the hexose monophosphate shunt. In case of very intense oxidative stress, this pathway may reach 90% of the glucose metabolism, in order to provide proper anti-oxidant defenses. (44, 60-61)

The RBC is a cell particularly prone to suffer oxidative damage, due to its function as an oxygen carrier and its poor repair mechanisms. The oxiHb suffers autooxidation *in vivo* at a rate of 0.5-3%, forming methHb that is not able to transport oxygen. If the antioxidant defences fail to detoxify the cell in superoxide radical (O_2^-) and hydrogen peroxide, other reactive oxygen species could be formed and lead to oxidative damages in the erythrocyte. (17)

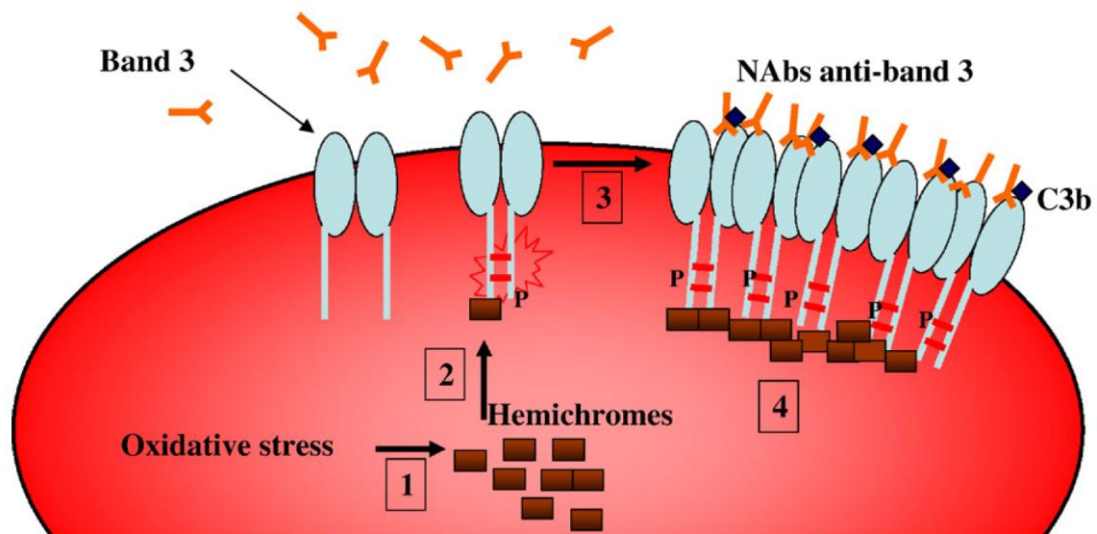
2.4.4. Band 3 neo-antigen

Several research studies focused on the mechanism underlying the RBC removal from the circulation, and on how senescent RBC are recognized by the macrophages within the spleen, liver and bone marrow. The erythrocyte has a membrane surface that is, usually, as all of our body cells, non-immunogenic. However, it has been identified a natural immunoglobulin (Ig) G anti-Band 3 in plasma. (54-59, 62)

The removal of senescent/damaged RBC must involve a change in components already present in the RBC membrane, as the mature erythrocyte does not present the capacity to synthesize or repair modified proteins. A senescent neo-antigen has been identified that is immunologically related to Band 3 protein, the major integral erythrocyte membrane protein. (70-72) The antigenic properties of Band 3 come from the modifications that it may suffer, by proteolytic cleavage, clustering, or even by exposure of unusual epitopes, triggering the binding of the specific natural auto-anti-Band 3 antibodies and complement activation, marking the cell to be removed from circulation. (Figure 12) (73-74)

The Band 3 is uniformly distributed in the RBC membrane. Along its life span, the RBC enzymatic activity reduces, limiting the RBC metabolisms and the antioxidant defences. When an oxidative stress develops within the RBC, Hb can undergo oxidation, followed by the linkage of oxidized hemoglobin to the cytoplasmic domain of Band 3, promoting the clustering of Band 3 (Figure 12). (73-74)

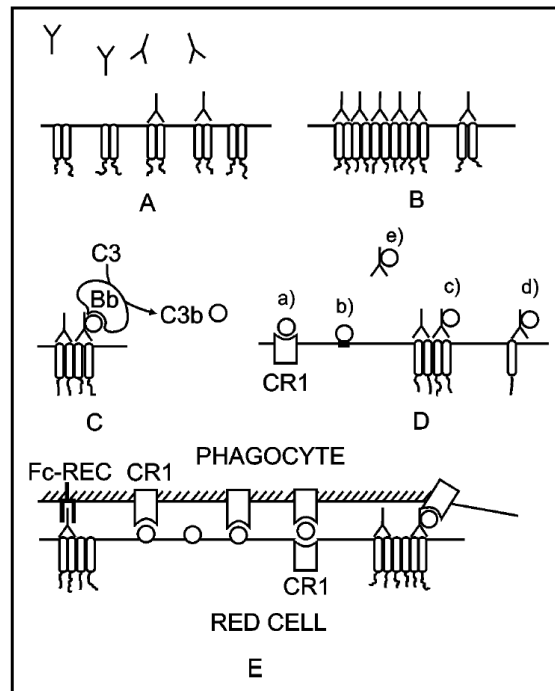
Figure 13. Hemoglobin oxidation and the promotion of band 3 aggregation and anti-band 3 antibodies binding.



NAb, natural antibodies. 1, Oxidative denaturation of hemoglobin leading to hemichrome formation; 2, Hemichrome binding to band 3 cytoplasmic domain causes their oxidative cross-linking through disulfide bonds and their Tyr phosphorylation; 3, Band 3 dissociation from cytoskeletal proteins and its clusterization; 4, Formation of large band 3/hemichromes clusters and opsonization by NABs and C3b. Adapted from Pantaleo *et al.* (57).

The anti-Band 3 IgGs that were linked to Band 3 by weak and easily dissociating bonds, in case of Band 3 aggregation, will link to Band 3 aggregates by more strong and permanent bonding. With Band 3 aggregation a neo-antigen is present in the membrane. The exact binding site is not perfectly known, but it may be on the polysaccharide residues of the carbohydrate chains of Band 3 (75-76) or result from an alteration in the C-terminal amino acids of the protein. (77-78)

The covalent binding of the anti-Band 3 antibodies will lead to complement activation, with deposition of the C3 factor in its active form (C3b). The deposition of C3b can be made directly in the RBC membrane or in the complement receptor. The complement activation will increase the RBC opsonization and recognition by macrophages. This recognition can be made through the recognition of the Fc part of the antibody by the macrophages Fc receptor or by the binding to C3b of macrophages complement receptor 1. (Figure 14) (59)

Figure 14. Band 3 aggregation opsonization and complement activation.

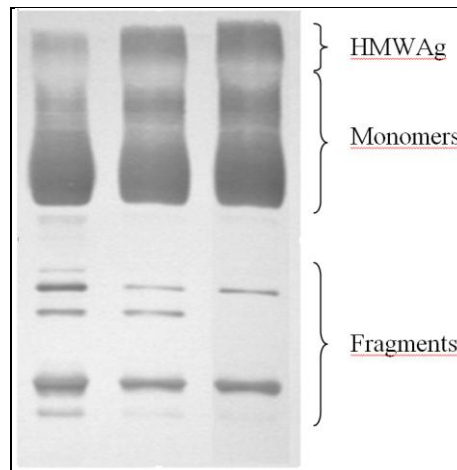
(A) Normal undamaged RBC.. (B, C) In senescent RBCs or after oxidant insult, aggregation of band 3 enhances binding of naturally-occurring anti-band 3 IgG and activates the alternative pathway of complement, leading to generation of C3b fragments. Clustered band 3 traps spontaneously generated complement fragments C3b. The binding prevents C3b from inactivation and allows formation of Bb convertase (Bb) that converts more C3 to C3b. (D) C3b can bind to complement receptor CR1 (a), bind covalently to RBC membrane (b), to band 3 clusters (c), to monovalently bound anti-band 3 IgG (d), or to other IgG (e). (E) Recognition of opsonized RBC occurs predominantly via CR1 or Fc receptor of the phagocyte. Adapted from Turrini *et al.* (59)

Besides the influence of the Band 3 aggregation on the binding of the anti-Band 3 antibodies and complement activation, it will also affect the RBC elasticity and deformability. As referred, Band 3 is uniformly distributed through the cellular membrane, creating well distributed points of anchorage for both proteins of the cytoskeleton and for the lipid fraction. When the distribution of Band 3 is uniform, and, therefore, the linkage of spectrin to Band 3, the membrane presents a great capacity to resist to pressure, due to the elongation of spectrin. However, when the aggregation of Band 3 occurs, the capacity of elongation of spectrin is limited. Moreover, the aggregation of Band 3 leads to other changes in the membrane, as some protein free areas will be formed and those strictly lipidic areas of the membrane will not have the usual protein anchorage to the membrane. Consequently, small portions of the membrane can be lost, leading to rounder, less flexible erythrocytes. (54-59)

When analysed by western blot, after SDS-PAGE of RBC membrane proteins (figure 15), the Band 3 presents as high molecular weight aggregates (HMWAg), monomers (Mon) and proteolytic fragments (Frag). (Kay, 1983 #387) As the RBC suffer damage/aging, the

percentage of HMWAg increases, Band 3 monomer (Mon) and total proteolytic fragments (Frag) decrease. As the RBC suffer damage/aging, the percentage of HMWAg increases, while there is a decrease in Mon and Frag, due to band 3 aggregation. (79)

Figure 15. Erythrocyte band 3 protein western-blot.



HMWAg, High molecular weight aggregates.

2.4.5. Removal of the senescent/damaged erythrocytes

The senescent or damaged erythrocyte accumulates the several changes, already described, marking the erythrocyte for removal from the circulation. This removal can occur by two ways:

- Extravascular hemolysis: the removal takes place outside of the blood vessels, in the spleen, liver or bone marrow; the erythrocyte is recognized by the macrophages of the reticulo-endothelial system (RES) and removed. Eighty to ninety percent of the extravascular hemolysis occurs in the spleen. (17)
- Intravascular hemolysis: the disruption of the RBC occurs inside the blood vessels. Only 10 to 20 % of the RBC are removed by this way. (43, 62, 80) Intravascular hemolysis is caused by the increasing rigidity of senescent RBC and by changes in the interaction with the vascular endothelium or by fibrin deposition. The removal of Heinz bodies (formed by oxidized Hb) in the spleen may lead to RBC fragmentation that may disrupt in blood circulation. (62, 80)

2.4.6. Hemoglobin catabolism

Following extravascular hemolysis, the Hb is divided in heme and globin chains. The heme group in the presence of heme oxygenase will be split in carbon monoxide, iron and biliverdine. The iron is reused in the bone marrow for the synthesis of new RBC or stored in the macrophages from the RES or in the hepatocytes. Biliverdine is converted by bilirubin reductase to bilirubin, which is a non hydrossoluble form. When released into the plasma it binds to albumin, forming the non-conjugated bilirubin-albumin complex that will transport bilirubin to the liver. In the liver, bilirubin undergoes glucurono conjugation becoming hydrossoluble; conjugated bilirubin is released into the biliary vesicle and eliminated in the stools. A small amount of conjugated bilirubin can be reabsorbed in the ileum and return to blood via the portal vein, being eliminated in the urine as urobilinogen. (62, 80)

When the RBC disrupt inside the vessels, the Hb is released in plasma. The free Hb conjugates with haptoglobin and is transported to liver. When haptoglobin is depleted, Hb is oxidized to metHb and dissociates non-enzymatically into heme and globin groups. The heme group can conjugate with albumin (methemalbumin) or with hemopexin (hemine-hemopexin), being transported to the liver to be metabolized. The iron from Hb will either go to bone marrow or will be stored in the hepatocytes. (62, 80)

2.5. Erythrocyte membrane changes in obesity

Several studies reported an altered profile of erythrocyte membrane Band 3 in different inflammatory conditions, known to be associated with oxidative stress, namely, in myocardial infarction (81), ischemic stroke (79), pregnancy and high competition physical exercise. (21)

In obesity, there are few studies about the changes occurring in RBC, namely in the RBC membrane. Some studies focused on the changes in the membrane lipid composition and found that the phospholipid content of the membranes appear to be related with insulin sensitivity (82) and also with oxidative stress. (Cazzola, 2004 #412; Elizondo, 2008 #395) Some of these changes seem to lead to a higher rigidity and lower deformability of the membrane. (83-84) Furthermore, the changes in the erythrocyte membrane composition

and deformability, in obesity, might interfere with the delivery of oxygen to tissues, leading to a certain degree of hypoxia. (83)

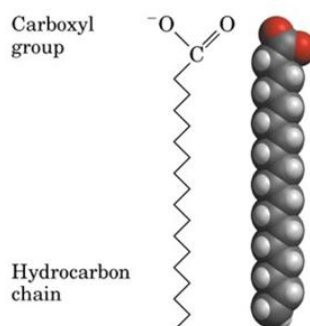
There is some controversy in literature regarding the effect of weight loss, in obesity, namely, if weight loss reduces oxidative stress and re-establishes normal physiology for the RBC membrane. Some authors found an improvement (84), while others did not. (85) The different results might be related to confounding factors, such as the magnitude of weight loss, the studied population and the study design. (86-87)

3. Fatty Acids

3.1. Introduction

Fatty acids (FA) are carboxylic acids present in biological systems, characterized by presenting a carboxylic acid group followed by a lateral carbon chain that can vary in size (Figure 16), from 3 (propyonic acid) to more than thirty carbons. (88)

Figure 16. Fatty acid structure.



Adapted from internet. (89)

FA are part of the class of lipids, a heterogeneous group that include hydrophobic biological molecules that share some physico-chemical and physiological characteristics. FA may exist free or linked to other molecules presenting, usually, a hydroxyl group; the bond formed is typically of the ester type, between the carboxylic group of the FA and the hydroxyl of the other molecule. (90)

When chemically combined with other molecules, the FA originate different lipid classes and are classified according to the linked molecule:

- Glycerides (mono, di or tri-glycerides) when linked to glycerol,
- Phospholipids (PL) when linked to glycerol or sphingosine,
- Cholesterol esters (CE) when linked to cholesterol. (90)

These different classes of lipids have important roles in the metabolism. As part of PL, the FA present a very important role in biological membranes. Different FA in membrane PL

modulate membrane fluidity, increasing with their content in unsaturated FA. Triglycerides (TG), on their turn, have an important role as energy reserve. (91-92)

One of the most important functions of lipids is their role as energy suppliers and depots. A gram of lipids can provide around 9 kcal, while a gram of proteins or glucids provide only 4 kcal. Furthermore, glycogen, the main form to store energy by glucids, in animals, is hydrated and adsorbs 2 grams of water per gram of glycogen. On the other hand, lipids, as triglycerides, can be stored in an almost anhydrous form, due to their hydrophobic characteristics. In global terms, and considering the lipid storage as anhydrous, each gram of hydrated glycogen can store 1.3 kilocalories (kcal) (4 kcal / (1g of glycogen + 2g of water)), compared with the 9 kcal/gram of triglycerides. By using lipids as energy reserve material, animals have a great energy storing capacity in a small body volume. (91-92)

3.2. Chemical structure

3.2.1. Ramification and length of the carbon chain

FA usually have an even number of carbons due to their synthesis being based in two carbon units (acetyl-CoA). Odd numbered FA are more common in bacteria. (90)

The structure of FA, according to their carbon chain, can be aliphatic, a linear straight carbon chain, or branched, when the carbon chain present one or more lateral chains. The majority of the natural FA are not branched. The aliphatic FA present in nature, usually, from 4 to 28 carbons. (90)

According to the length of the carbon chain, the FA can be classified as (93):

- Short-Chain FA – up to 6 carbons
- Medium-Chain FA – from 6 to 12 carbons
- Long-Chain FA – from 12 to 22 carbons
- Very Long-Chain FA – over 22 carbons

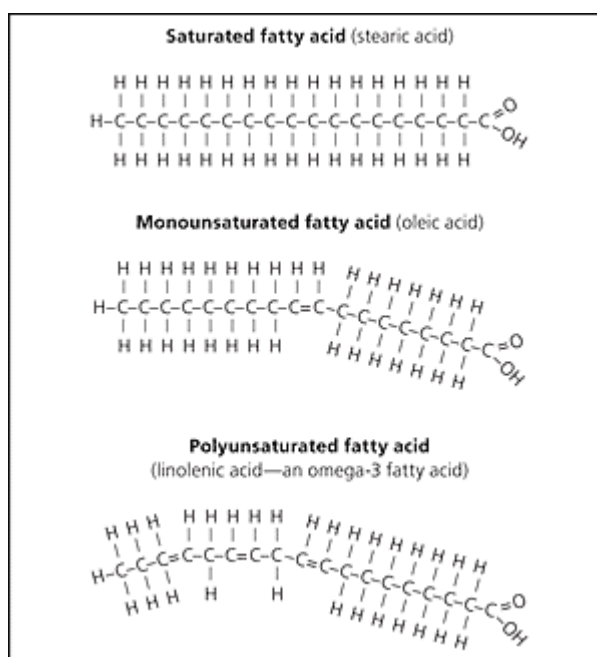
3.2.2. Unsaturation

Each carbon atom can present up to 4 bonds with other atoms. These bonds may be with 2 to 4 different atoms, depending on the existence of double or triple inter atomic bonds. In acetylene, for example, the two carbons are linked by a triple bond leaving one available bond in each carbon that is filled by hydrogen (C_2H_2). FA are unsaturated or saturated, according to the presence or not of double or triple bonds between carbons. (90)

A saturated FA (SFA) present no double bond between its carbons and, therefore, each of the lateral chain carbons are linked to the two adjacent carbons and to two hydrogen atoms (except for the terminal carbon of the chain that is bound to one carbon and to 3 hydrogen atoms) (Figure 17). (90)

UFA can be classified as monounsaturated FA (MUFA) and polyunsaturated FA (PUFA) (Figure 17). There is also a special term for PUFA with carbon chains longer than 22 carbons - Very Long PUFA (VLPUFA). (84)

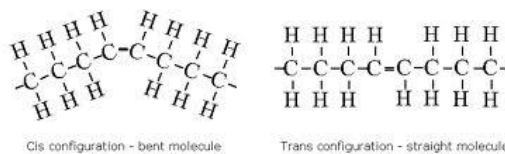
Figure 17. Types of fatty acid according to the number of unsaturation in the lateral carbon chain.



Adapted from internet. (94)

The double bonds of unsaturated FA are more rigid and, as they fix the position of the groups in the carbons forming the bond. If the two hydrogens are in the same side of the double bond we have a *cis* structure and, if the hydrogens are in opposite sides of the chain, we have a *trans* double bond (Figure 18). The majority of the double bonds found in nature are of the *cis* type. *Trans* double bonds can also result from human processing of fats (hydrogenation/hardening of vegetal oils). (95)

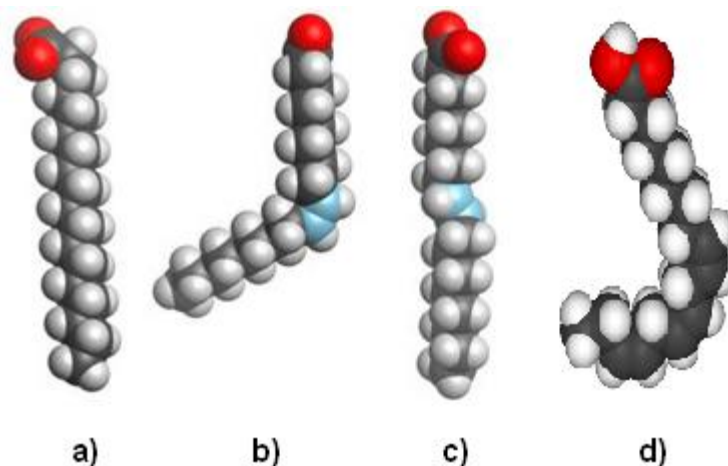
Figure 18. *Cis* and *trans* fatty acid configuration of double bonds in fatty acids.



Adapted from internet. (96)

In the case of *cis* double bonds, the fact that both the larger groups are in the same side of the double bonds, causes a steric interaction, making the carbon chain to curve a little. For every *cis* double bond added, the chain curves a little more. This steric effect is the reason why oleic acid (C18:1n9*cis*) has a small turn in the chain, while linolenic acid (C18:3n3*cis,cis,cis*) has a pronounced hook aspect. *Trans* double bonds, due to reduced steric interaction, present an almost linear chain as is shown by elaidic acid (18:1n9*trans*) (Figure 19). (88)

Figure 19. Fatty acids spatial impact of trans and cis double bonds in the carbon lateral chain.



a) Stearic acid (C18:0); b) Oleic acid (C18:1n9cis); c) Elaidic acid (C18:1n9trans), d) Linolenic acid (C18:3n3cis,cis,cis).

Adapted from internet. (97-98)

As a result of the curvature present in the chains of *cis* unsaturated FA, they cannot interact with neighbour chains in the same way SFA do. Because of this, SFA present higher melting points than MUFA, and MUFA present higher ones than PUFA (Figure 20). *Trans* FA present a straighter chain and, thus, have intermolecular interaction similar to SFA and, consequently, higher melting temperatures when compared to their unsaturated isomers (Figure 20). (88)

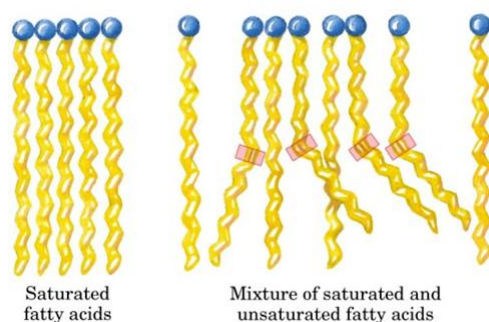
Figure 20. Saturated, Monosaturated and Polyunsaturated fatty acids, their structure and melting temperature.

FATTY ACIDS					
Saturated			Unsaturated		
Formula	Common Name	Melting Point	Formula	Common Name	Melting Point
$\text{CH}_3(\text{CH}_2)_{10}\text{CO}_2\text{H}$	lauric acid	45 °C	$\text{CH}_3(\text{CH}_2)_5\text{CH}=\text{CH}(\text{CH}_2)_7\text{CO}_2\text{H}$	palmitoleic acid	0 °C
$\text{CH}_3(\text{CH}_2)_{12}\text{CO}_2\text{H}$	myristic acid	55 °C	$\text{CH}_3(\text{CH}_2)_7\text{CH}=\text{CH}(\text{CH}_2)_7\text{CO}_2\text{H}$	oleic acid	13 °C
$\text{CH}_3(\text{CH}_2)_{14}\text{CO}_2\text{H}$	palmitic acid	63 °C	$\text{CH}_3(\text{CH}_2)_7\text{CH}=\text{CH}(\text{CH}_2)_7\text{CO}_2\text{H}$	elaidic acid	47 °C
$\text{CH}_3(\text{CH}_2)_{16}\text{CO}_2\text{H}$	stearic acid	69 °C	$\text{CH}_3(\text{CH}_2)_4\text{CH}=\text{CHCH}_2\text{CH}=\text{CH}(\text{CH}_2)_7\text{CO}_2\text{H}$	linoleic acid	-5 °C
$\text{CH}_3(\text{CH}_2)_{18}\text{CO}_2\text{H}$	arachidic acid	76 °C	$\text{CH}_3\text{CH}_2\text{CH}=\text{CHCH}_2\text{CH}=\text{CHCH}_2\text{CH}=\text{CH}(\text{CH}_2)_7\text{CO}_2\text{H}$	linolenic acid	-11 °C
			$\text{CH}_3(\text{CH}_2)_4(\text{CH}=\text{CHCH}_2)_4(\text{CH}_2)_2\text{CO}_2\text{H}$	arachidonic acid	-49 °C

Adapted from internet. (99)

The increase of the melting point with increasing degree of unsaturation is important for membrane and fat rheology. For example, in case of low temperatures, the *cis* unsaturated FA will not be as closely packed as SFA and *trans* FA and, thus, the membrane fluidity is greater (Figure 21). (83-84)

Figure 21. Packing of saturated fatty acids and of a mix of saturated and unsaturated fatty acids.



Adapted from internet. (89)

This is also the reason why vegetal oils from tropical countries are, usually, richer in SFA, and, therefore, are semi solid or solid at room temperature, while in colder countries there is an increase in the proportion of unsaturated FA, thus, preventing the plant to freeze. PL have also impact in the fluidity of the cellular membrane, with unsaturations increasing fluidity. This is one of the reasons why cold water fishes present in their membranes a higher proportion of PUFA. (83-84)

3.3. Nomenclature

Different nomenclature systems are used for FA. Similarly to other organic compounds, there is the official International Union of Pure and Applied Chemistry (IUPAC) systematic nomenclature and the trivial nomenclature. The IUPAC nomenclature is unambiguous and defines clearly the molecule, however, sometimes, it is too wordy. The trivial nomenclature has also a degree of unambiguousness but is less clear about structural and conformational aspects. (88, 100)

Different notations, to describe the carbon position in the chain, also exist:

The Δ , or Δ -x (delta *minus* x) notation: double bonds are pointed according to the position in their FA chain, counting from the carboxylic terminal. The spatial orientation of the double bond is described by adding the prefix *cis* or *trans* before “ Δ ”. E.g. *cis*, *cis* Δ 9,12octadienoic acid for trivial linoleic acid. (88, 100)

The ω , also known as ω -x (omega *minus* x) or n-x (n *minus* x) notation: double bonds are pointed counting the carbon from the metil carbon (terminal carbon of the chain – also called ω (the last letter of the Greek alphabet) or n carbon). Strictly, it means “the last carbon minus “x” carbons until the double bond”. It is from this notation that originates the well known term “omega 3 acids” – a family of FA that have similar biological roles and that share the characteristic of presenting a double bond in the 3rd carbon counting from the metil end of the FA chain. E.g. 18:3n3 for linolenic acid, a FA with 3 unsaturations from the omega 3 family. (88, 100)

The widely use of the notations n-x or ω -x is due to the physiological and metabolic connections between the FA in the same ω family. In fact, this last double bond is energetically and physiologically very stable, since the majority of the interactions between the FA and other compounds is done by the carboxyl end (e.g. with glycerol) or with the double bonds in the middle of the carbon chain (e.g. the eicosanoids synthetic pathway). Also, the FA from the same family are, in some way, interchangeable and share metabolic pathways and functions. (101)

The lipid number is another type of notation used. It specifies the number of carbons in the molecule, followed by the number of double bonds, e.g. 18:1, for a FA with 18 carbons and one unsaturation. It is not very precise, as 18:1 can indicate either the oleic acid or the elaidic acid (oleic *trans* isomer). This is the reason why it is usually used together with the Δ or ω notation, e.g. 18:1n9c and 18:1n9t for oleic and elaidic acid, respectively. (88, 100)

Figure 22 present some examples for fatty acid nomenclature using different notations.

Figure 22. Trivial, systematic and abbreviations used in FA nomenclature.

Name		Abbreviation	
Trivial	Systematic (IUPAC)	Carboxyl carbon	Metil Carbon
Palmitic	Hexadecanoic	16:0	16:0
Stearic	Octadecanoic	18:0	18:0
Oleic	9-octadecanoic	18:1 Δ 9 cis	18:1 ω 9
Elaidic	9-octadecanoic	18:1 Δ 9 $trans$	18:1 ω 9
Linoleic	9,12-octadecadienoic	18:2 Δ 9,12	18:2 ω 6
α -Linolenic	9,12,15-octadecatrienoic	18:3 Δ 9,12,15	18:3 ω 3
γ -Linolenic	6,9,12-octadecatrienoic	18:3 Δ 6,9,12	18:3 ω 6
Arachidonic	5,8,11,14-eicosatetraenoic	20:4 Δ 5,8,11,14	20:4 ω 6
Docosahexaenoic (DHA)	4,7,10,13,16,19-docosahexaenoic	22:6 Δ 4,7,10,13,16,19	22:6 ω 3

Double bonds are considered *cis* and unconjugated if the contrary is not referred. In the present thesis the different notations are used.

3.4. Synthesis

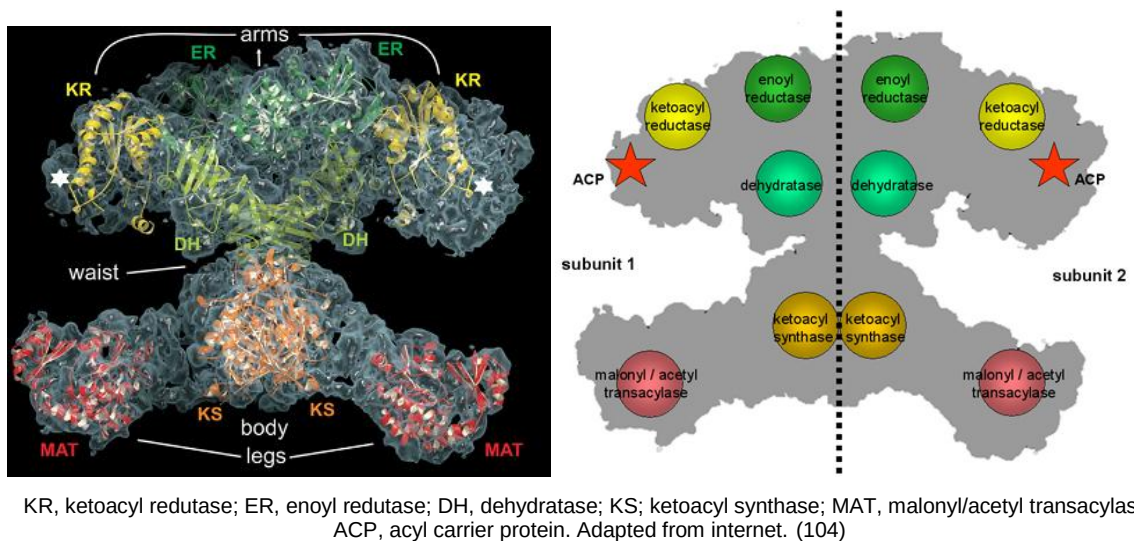
Human body is able to synthesize FA, except essential FA (EFA), that are necessary for many of our body functions and structures. This process is also essential for lipogenesis, as FA are part of different lipid classes. In mammals, FA are produced mainly in the liver, but also in the breast tissue of lactating women and in the adipose tissue. (102)

FA synthesis occurs in the cytosol, however acetyl-CoA units arise from mitochondria. There are two main enzymes involved in FA synthesis in mammals, the acetyl-CoA carboxylase and the FA synthase. (103)

The FA synthase is a cytosolic polymeric protein that contains the different enzyme functions necessary to produce saturated FA until 16 carbons length (palmitic acid), starting from acetyl-CoA and malonyl-CoA. The difference between the FA synthesis in mammals and bacteria is that in the latter the FA synthesis is catalyzed by 6 different enzymes, while in mammals there is only one enzyme formed of two homo-monomers – FA synthase. This large multi-enzyme protein was probably been originated by gene fusion. (103)

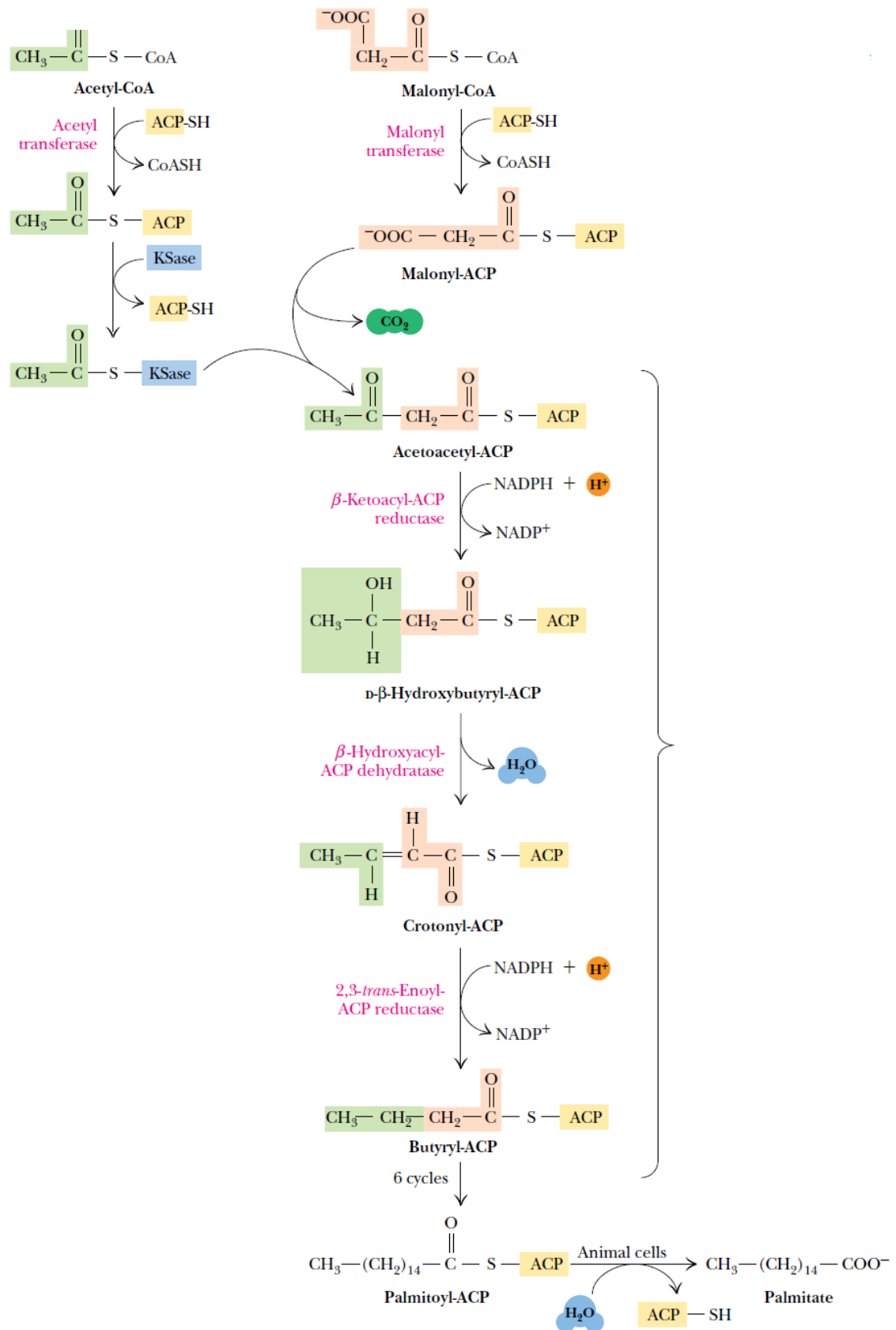
Figure 23 presents the FA synthase structure and the organization of the different catalytic points.

Figure 23. FA synthase structure and the organization of the different catalytic points.



The synthesis beginning with two molecules of acetyl-CoA, one of them is carboxylated to malonyl-CoA by acetyl-CoA carboxylase with the hydrolysis of one ATP. The FA synthase binds the two molecules and transfer the malonyl-CoA to the acetyl-CoA adding two carbon units to acetyl-CoA and producing a 4 carbon molecule (butyl-CoA). In this process are involved many subunits of FA synthase, with different enzymatic activities, by this order: acetyl-CoA:Acyl Carrier Protein (ACP) transacylase, malonyl-CoA:ACP transacylase, 3-ketoacyl-ACP synthase, 3-ketoacyl-ACP reductase, 3-ketoacyl-ACP dehydrase and, finally, enoyl-ACP reductase. The growing FA is constructed through continuous linkage of 2 carbon units, coming from malonyl-CoA (Figure 24). (91-92)

Figure 24. Fatty acid synthesis reactions performed by fatty acid synthase.

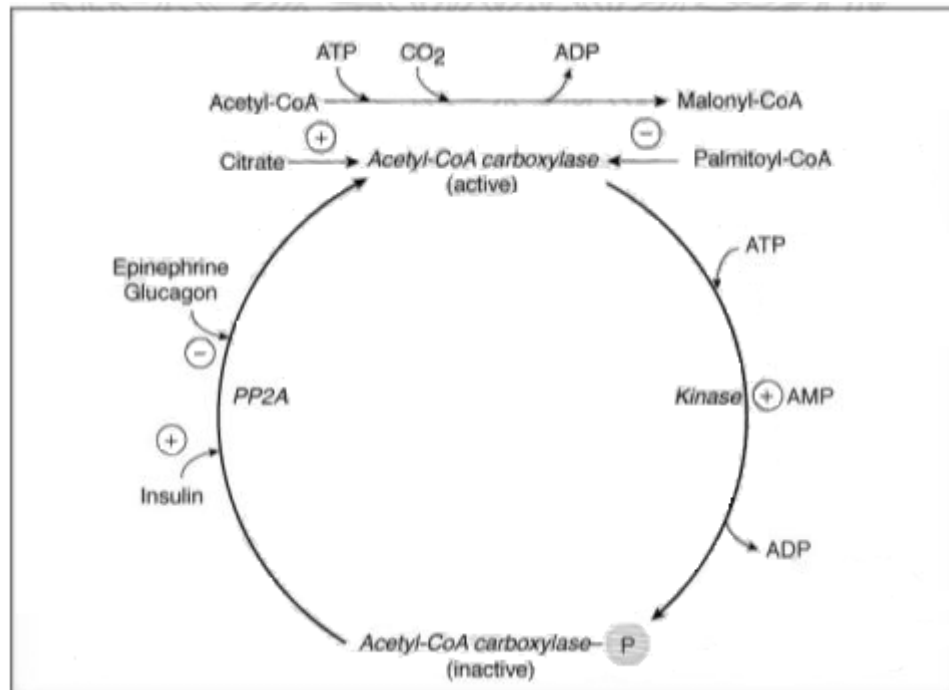
ACP, Acyl Carrier Protein. Adapted from *Biochemistry*. (105)

The process does not have to go until the production of palmitic acid, smaller FA can be produced by early chain termination. Production of FA with longer chains involves enzymes present in the endoplasmic reticulum (ER). (103)

3.4.1. Synthesis regulation

Regulation: The rate limiting step in the FA synthesis is the carboxylation of acetyl-CoA to malonyl-CoA by acetyl-CoA carboxylase. This enzyme is regulated by phosphorylation and also by allosteric binding (Figure 25): (106)

- Allosteric binding:
 - Inhibition: The enzyme is inhibited allosterically by the palmitic acid, a classical feedback inhibition. When inactive, the enzyme is in a protomer form. (107)
 - Activation: Citric acid activates the FA synthase. Citric acid is the result of the reaction between oxaloacetate and acetyl-CoA and is a part of Krebs cycle. If there is too much citric acid, it indicates that it is not necessary more acetyl-CoA to move the cycle and the exceeding acetyl-CoA can be used to build FA (that can be used for energy storage or for other functions). (106) In this state the enzyme forms a multimeric filamentous complex. (107)
- Phosphorylation: When energy is necessary or glucose levels are low, glucagon, nor-epinephrine and epinephrine lead to the phosphorylation and consequent inactivation of acetyl-CoA carboxylase by a G protein coupled and AMPc involving mechanism. This stops FA synthesis and leaves acetyl-CoA free to be used as energy source. Also, when the cell's levels of AMP increases, meaning a decrease in the energy supply, an AMP activated kinase may phosphorylate and, thus, block the acetyl-CoA carboxylase. Insulin, as an anabolic hormone, however, leads to the de-phosphorylation and activation of acetyl-CoA carboxylase through a phosphatase and also by breaking down AMPc. Insulin promotes lipogenesis. (106)

Figure 25. Acetyl-CoA carboxylase regulation

Adapted from Biochemistry. (108)

FA synthase is subject to transcription regulation. In the liver, insulin stimulates the transcription through a mechanism involving upstream stimulatory factors (USF) and sterol response element binding protein 1 (SREBP-1), thus, stimulating lipogenesis. In the adipocytes, leptin inhibit transcription, by blocking the SREBP-1 action, and so preventing excess of fat storage. (109)

3.4.2. Beyond palmitic acid

For human metabolism FA with longer chains and higher unsaturation degrees than the palmitic acid are also necessary. A great part of these FA is obtained directly from diet. Some FA, the EFA, have to be obtained from foods, since humans do not present the necessary enzymatic machinery for their production. (91-92)

Both the enzymes necessary for further elongation and introduction of unsaturations are present in the ER. These enzymes can interact and produce a variety of FA through intercalating the action of elongation and unsaturation. (91-92)

3.4.3. Elongation

A class of enzymes, the FA elongases, performs elongation of FA beyond the 16 carbons of the palmitic acid produced by the FA synthase. These enzymes are present in the ER and will elongate the FA chains two carbons at a time, by adding residues of malonyl-CoA in a very similar way of that of FA synthase. The difference is that, in this case, different enzymes, instead of a multifunctional protein, catalyze the elongation. (91-92)

3.4.4. Desaturation

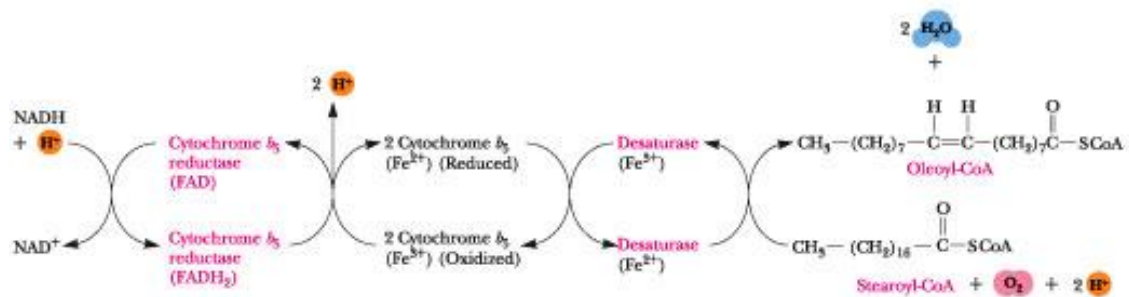
FA desaturation is also performed by enzymes present in the ER, the desaturases. These enzymes are responsible for the introduction of double bonds in FA coming either from endogenous synthesis or from diet. (91-92)

The human desaturases are included in four different families according to the carbon atom position, counting from the carboxylic end of the FA chain, where the double bonds are introduced. Thus, the desaturases are called $\Delta 4$, 5, 6 and 9. (91-92)

There are EFA because humans cannot introduce desaturation in carbons after the tenth carbon counting from the carboxylic end (the ninth carbon is the limit). Therefore, it is necessary to obtain these FA from diet, e.g. linoleic acid (desaturation in carbons 9 and 12) and linolenic acid (desaturation in carbons 9, 12 and 15). (110)

The desaturation of FA involves 3 enzymes: NADH-cytochrome (CYP) b5 reductase, CYP b5, and the desaturase itself (Figure 26). The desaturases have 2 iron atoms in the active site that are extremely important for the enzyme function. In the desaturation process two electrons and hydrogens are removed from each carbon atom where the double bond will be introduced and are transferred to oxygen, producing water. (91-92)

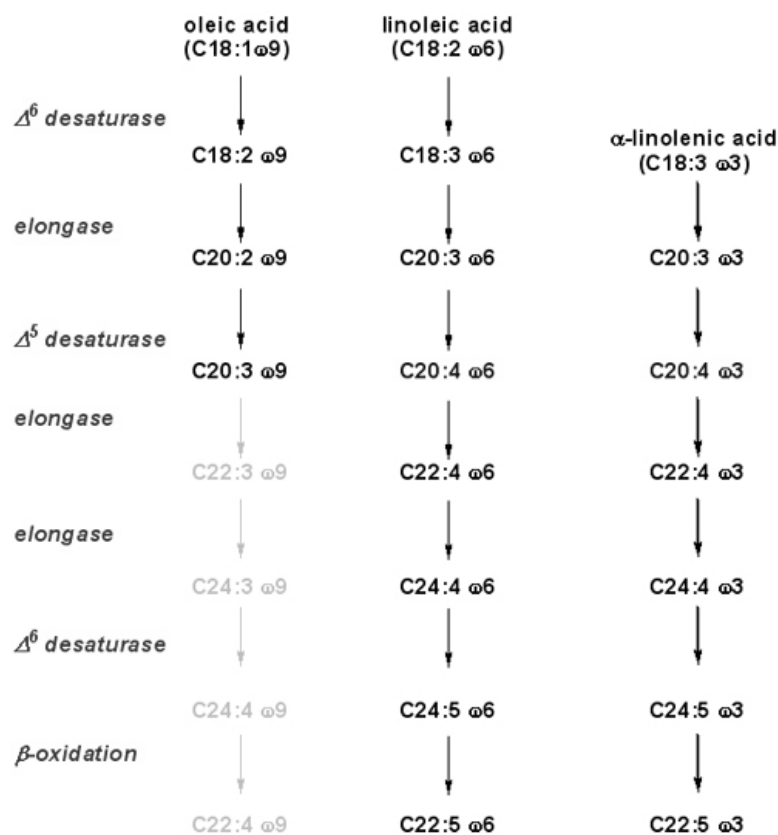
Figure 26. Production of oleoyl-CoA from steroyl-CoA through the introduction of a double bon in the carbon chain.



Adapted from *Biochemistry*. (105)

The FA of different classes (SFA, UFA, ω families) compete for the same enzymes responsible for elongation and desaturation. Dietary FA also compete with endogenous ones. In this way, the excess of a class of FA have effect on the synthetic pathways of the other classes. (91-92) Figure 27 resumes the enzymes involved in FA from ω 3, 6 and 9 families.

Figure 27. Enzymes involved in the synthesis of fatty acids from the 3 omega families.



In lighter color is shown a less important pathway. Adapted from internet. (104)

3.5. Fatty acids as energy

3.5.1. Lipolysis

FA can be used by the organism as an energy supply. The FA used for this purpose, usually, come from TG hydrolysis. The glycerol released after TG hydrolysis can also be used to produce energy through glycolysis. (91-92)

In adipocytes, as well as in other cells that accumulate lipids, when the glucose levels are low, through glucagon, or when there is a metabolic demand for energy, through epinephrine, for example, an enzyme, the hormone sensitive lipase (HSL) is activated and starts TG hydrolysis. HSL breaks TG in monoglycerides and free FA (FFA). The FFA can then be used for energy production through oxidation. (111) The preferential substrate for HSL appears to be, in fact, diglycerides. Adipocyte TG lipase may have an important role in transforming TG in diglycerides and, thus, facilitating HSL function. Monoglycerides are hydrolyzed by monoglyceride lipase. (112)

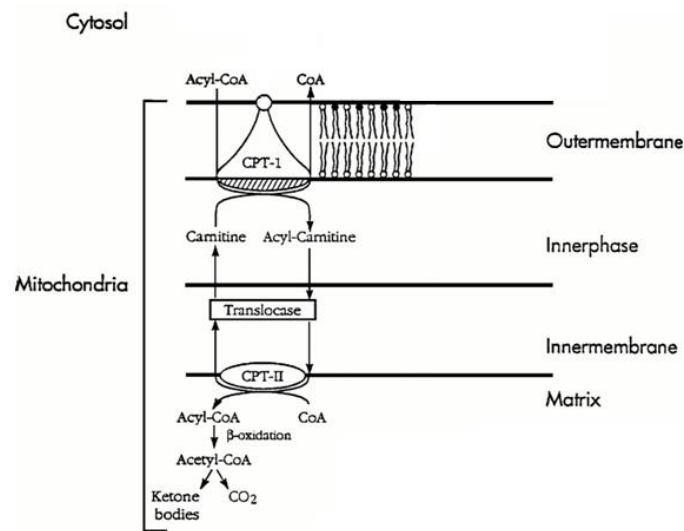
HSL is activated when phosphorylated by protein kinase A. After the binding of hormones, like glucagon and epinephrine, to the membrane receptor and through a G protein coupled mechanism, the levels of AMPc increases and activates the protein kinase A. In contrast, insulin dephosphorylates and inactivates HSL by protein phosphatase A2. Insulin may also activate a phosphodiesterase enzyme that breaks AMPc, thus, reducing protein kinase A activation. (91-92)

3.5.2. β -Oxidation

β -oxidation is the main form of transformation of FA into energy. This process takes place in the mitochondrial matrix. By β -oxidation FA will be shortened by 2 carbons units at a time, resulting from each cycle acetyl-CoA that can be used in the Krebs cycle. β -oxidation is a process that is somehow opposite to the FA synthesis. In fact, regarding the endogenous lipids, the first 2 carbons unit to be removed is the one that was added the last in the synthesis pathway. (91-92)

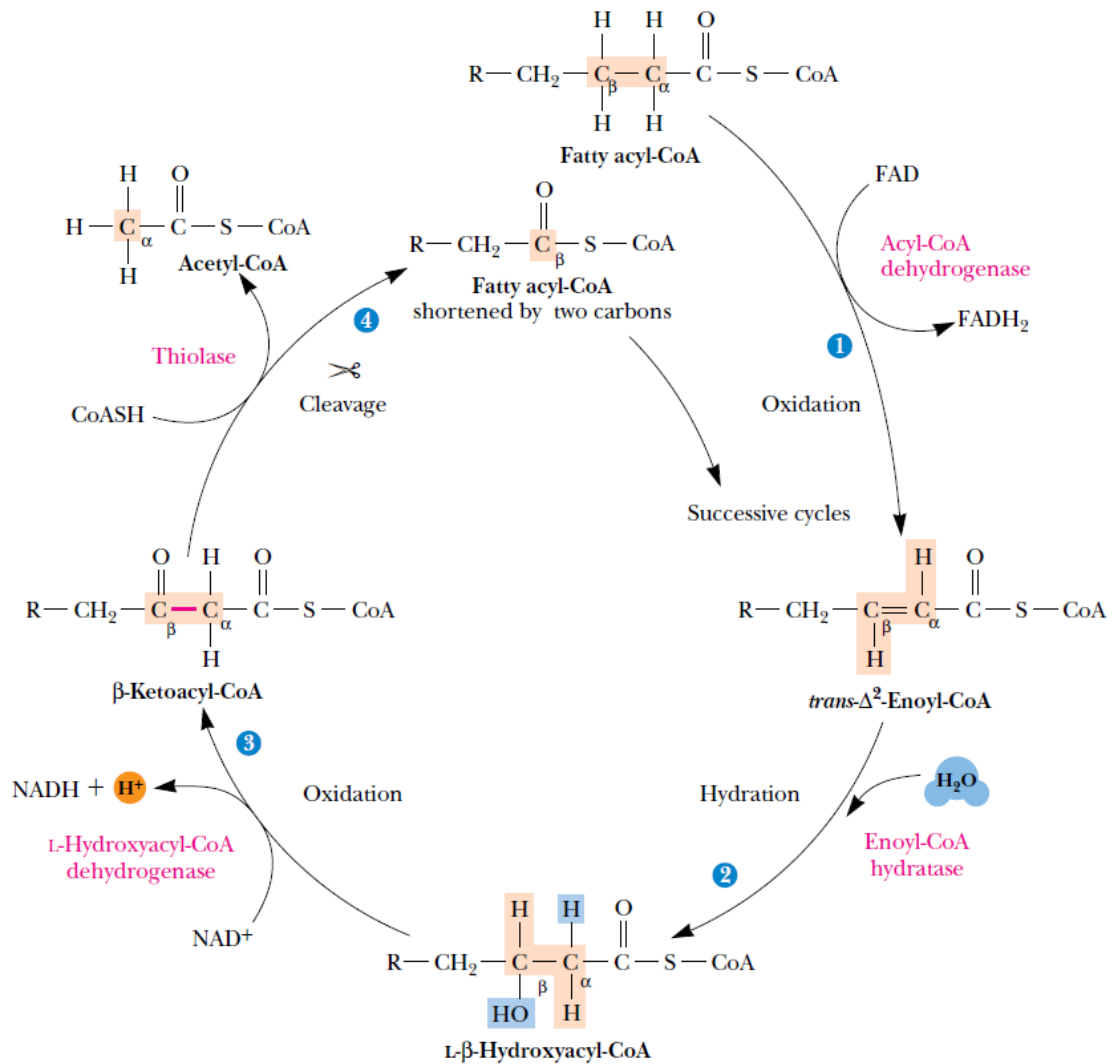
FA of shorter chain, until 12 carbons, can enter the mitochondria directly. FA with larger chains have to be transformed in their acyl-CoA derivate, at the expense of ATP hydrolysis to adenine monophosphate (AMP). The acyl-CoA can then pass through the outer mitochondrial membrane. To pass the inner mitochondria membrane, and to enter the matrix, the acyl-CoA is transferred to carnitine by carnitine-palmitoyl transferase (CPT) 1. Then, in the matrix, the acyl-carnitine is transferred back to CoA by CPT-2 and can be subjected to β -oxidation (Figure 28). The cytoplasmic and mitochondrial pools of CoA are independent. CPT-1 is thought to be the rate limiting enzyme of FA β -oxidation. (113)

Figure 28. Mechanism of entrance of fatty acid acyl-CoA to enter the mitochondrial matrix.



CPT, Carnitine-palmitoyl transferase; CoA, Coenzyme A. Adapted from Kim *et al.* (114)

β -oxidation reactions are, by this order: 1) dehydrogenation by acyl-CoA dehydrogenase, with the production of FADH_2 ; 2) hydration by enoyl-CoA hydratase; 3) dehydrogenation by 3-hydroxyacyl-CoA dehydrogenase, with the production of NADH, and, finally, 4) it is originated a double keto-derivative that will be broken by thiolase, originating a acyl-CoA two carbons shorter and one acetyl-CoA (Figure 29). Even numbered FA are broken by β -oxidation until two units of acetyl-CoA, while odd numbered FA originate one molecule of propionyl-CoA and one of acetyl-CoA. (91-92)

Figure 29. Fatty acid β -oxidation cycle.Adapted from *Biochemistry*. (115)

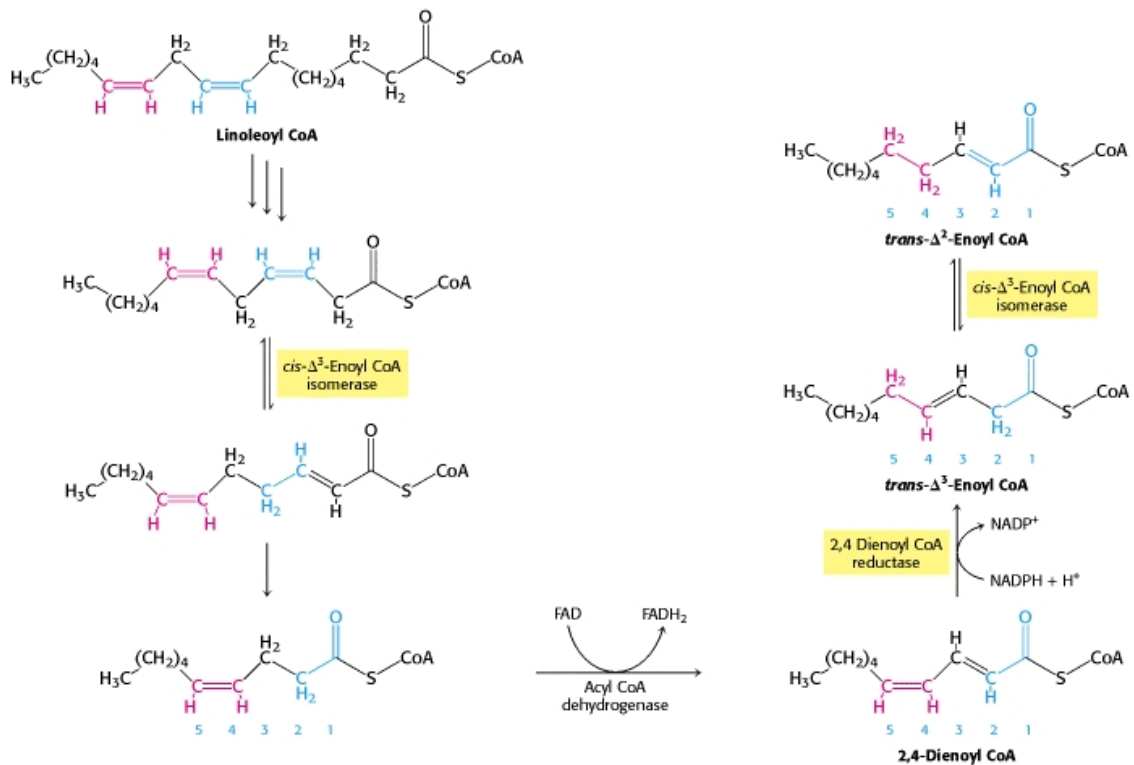
After carboxylation propionyl-CoA (at the expense of one ATP) originates methylmalonyl-CoA that can be rearranged to succinyl-CoA by an enzyme that has vitamin B12 as a co-factor. Succinyl-CoA can enter the Krebs cycle or be used as a precursor in the synthesis of the heme group. This is one of the reasons why vitamin B12 deficiency can lead to anemia. (91-92)

Unsaturated FA undergo to β -oxidation in the same way as SFA, with particular variations. Unsaturated FA with a double bond in an odd numbered carbon will originate, by consecutive breaking cycles, an acyl derivative with the double bond on carbon 3. To proceed with β -oxidation, it is necessary to perform an isomerization, by Δ^2 -enoyl-CoA

isomerase, and transfer the double bond to carbon 2. No FADH_2 is produced in this process. (91-92)

Unsaturated FA with double bond in an even numbered carbon generate, by consecutive oxidation, an acyl-derivate with a double bond in the carbon 4. The introduction of the double bond by dehydrogenation in the carbon 2 will produce conjugated double bonds (carbons 2 and 4) that will trigger the following step of β -oxidation, hydration, to occur at carbon 4 instead of in right carbon, carbon 2. Consequently, after introduction of the double bond in carbon 2, the action of a 2,4-dienoyl-CoA reductase, using NADPH as a co-factor, will reduce the intermediate to form an acyl-CoA with a double bond in carbon 3. The following steps of β -oxidation will follow those of odd numbered FA. The β -oxidation of linoleic acid (unsaturation in carbon Δ 9 and 12) is presented in figure 30 and involves the two cases discussed for the unsaturation position. Due to the use of NADPH or to not producing FADH_2 , unsaturated FA β -oxidation originates less energy than that originated by SFA with the same carbon chain size. (91-92)

Figure 30. β -Oxidation of unsaturated fatty acid Linoleic acid.



Adapted from *Biochemistry*. (92)

3.5.3. Peroxisomal oxidation

FA with very long chains cannot enter the mitochondria, even if linked to CoA and carnitine. They are subjected to a prior oxidation, called peroxisomal-oxidation. This process takes place in peroxisomes, a small cytoplasmic organelle. To enter the peroxisomes FA do not need to be linked to CoA, they can diffuse through the organelle membrane. (91-92)

Inside the peroxisome the FA will be broken in consecutive fragments of 2 carbons, in a process very similar to β -oxidation. However, there is no production of FADH_2 as final product, as the hydrogen acceptor is oxygen leading to the formation of hydrogen peroxide. (91-92)

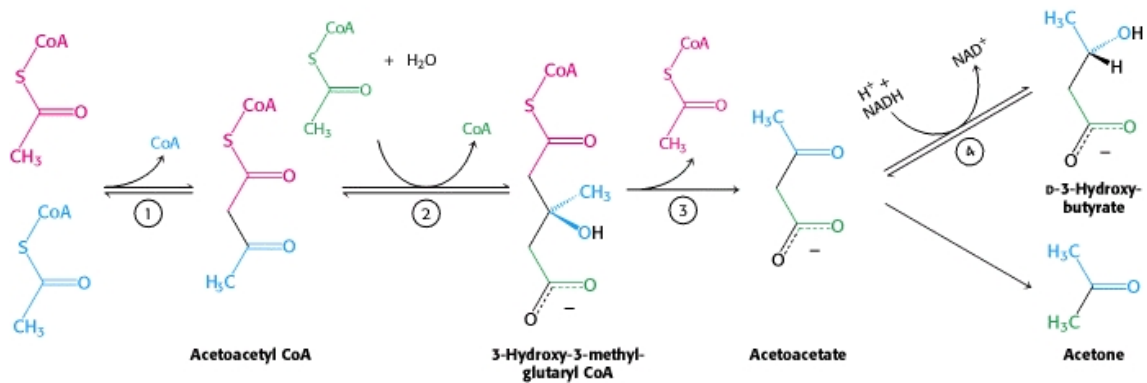
Peroxisomal oxidation is not complete as the peroxisomal thiolase will not hydrolyze acyl-CoA smaller than 8 carbons. The 8 carbons products can proceed to the mitochondria for β -oxidation. (91-92)

3.5.4. Other oxidation pathways

Other oxidation mechanisms are α -oxidation and ω -oxidation. The first one is used for branched FA, and the ω -oxidation occurs in the liver smooth ER. Usually, ω -oxidation is used for detoxification processes and accounts for around 15% of FA oxidation; however, it can grow to higher values, in case of needs, as occurs in β -oxidation defects. (91-92)

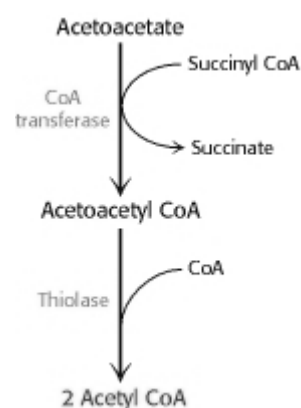
3.5.5. Ketone bodies

Part of the acetyl-CoA generated by FA oxidation in the mitochondria can be used to produce the so called ketone bodies: acetoacetate and β -hydroxybutyrate. These compounds are produced through the condensation of 3 acetyl-CoA units that originates 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). The enzyme HMG-CoA lyase reduces HMG-CoA and produces acetoacetate and acetyl-CoA (Figure 31). (116)

Figure 31. Ketone bodies synthesis.

1) Thiolase; 2) Hydroxymethylglutaryl-CoA (HMG-CoA) synthase; 3) HMG-CoA Lyase; 4) β -Hydroxybutyrate dehydrogenase. Adapted from *Biochemistry*. (92)

Ketone bodies can be used by organs, as the muscles and the heart, to produce energy. The brain can also use ketone bodies as energy supply in case of fasting and low availability of glucose, which is the brains' major energy source. Acetoacetate, after entering the mitochondria combines with succinyl-CoA forming succinate and acetoacetyl-CoA. The acetoacetyl-CoA can be cleaved by thiolase in two acetyl-CoA molecules that may enter the Krebs cycle and, ultimately, be used to produce energy for the cell (Figure 32). (116)

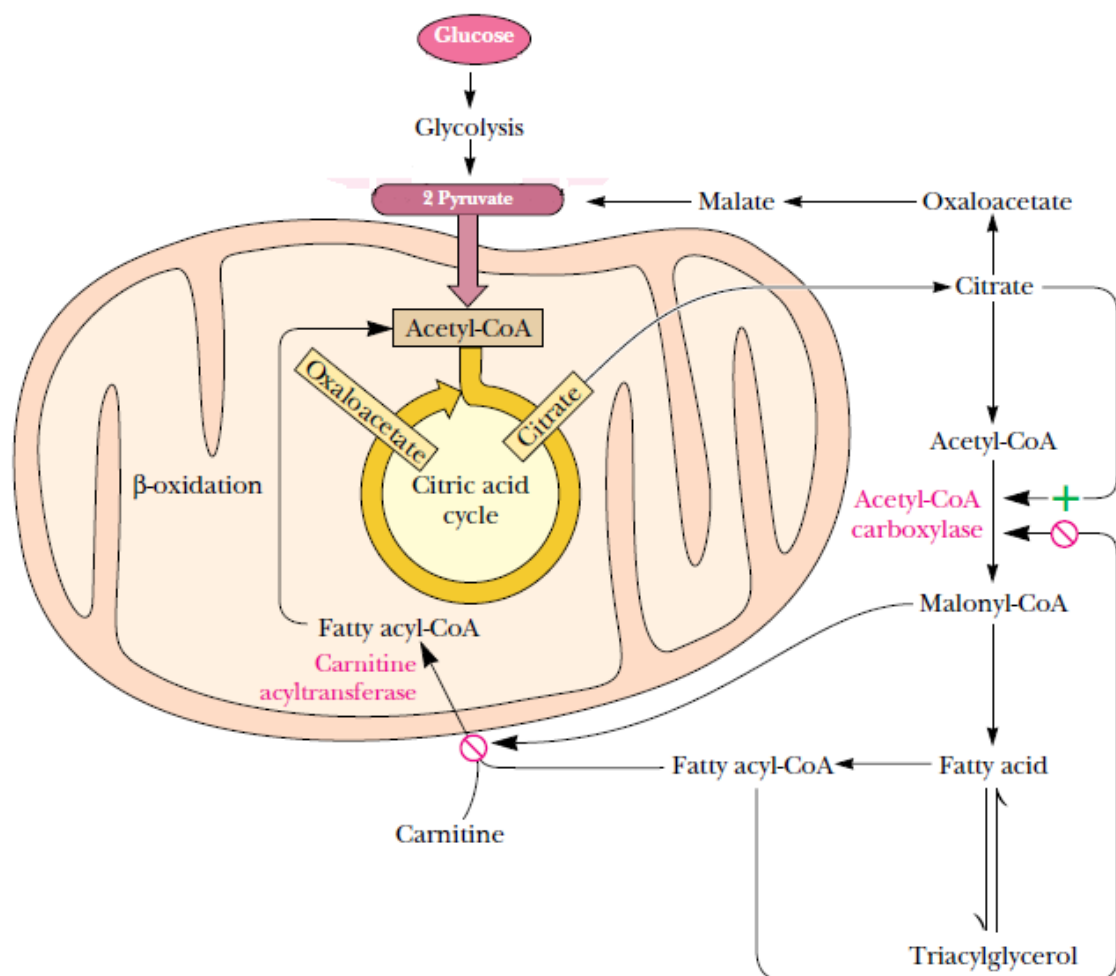
Figure 32. Transformation of ketone bodies in acetyl CoA.

Adapted from *Biochemistry*. (92)

3.6. Linkage to glucidic metabolism

The units of acetyl-CoA necessary to the FA synthesis come from the mitochondria and are, mostly, a product of glycolysis. Pyruvate, coming from glycolysis, is transformed in acetyl-CoA by pyruvate-dehydrogenase inside the mitochondria. To leave of mitochondria the acetyl-CoA combine with oxaloacetate, producing citric acid that passes to the cytosol, where it regenerates acetyl-coA and oxaloacetate. Oxaloacetate, in the cytosol, is reduced to malate and can than go through two different ways: 1) returns to mitochondria; 2) can be dehydrogenated by the malic enzyme with the production of NADPH, important co-enzyme for FA synthase reduction steps. Thus, the excess of acetyl-CoA produced by glycolysis can be used to the synthesis of FA (Figure 33). (102)

Figure 33. Link between Fatty acid synthesis, β oxidation and glucose metabolism.



Adapted from *Biochemistry*. (105)

The production of NADPH by the malic enzyme is not enough for FA synthesis. The biggest part of this co-enzyme comes from the pentose-phosphate pathway, another example of the connection between lipid and glucidic metabolism. (102)

Besides these links an important point of connection is the common hormonal control shared by glucidic and lipid metabolism, especially through insulin, but also by other hormones like glucagon, epinephrine, etc. These hormones will, as already shown, modulate both FA catabolism and synthesis, contributing to a delicate balance that enables our body to react and adapt to different environmental conditions. (91-92)

3.7. Dietary fatty acids

Although our organism has the capacity to synthesize several FA, exception made for the EFA, the majority of our FA come from dietary fats. Fats are present either in animal and vegetal derived foods, e.g. lard and butter, as animal derived fat, and seeds oils, as vegetal fats. In the Mediterranean diet, the lipids coming from some specific group of foods, as fishes and olive oil, are important. (117)

Different types and proportions of FA are found in different types of food, and the recommended Daily intake varies between FA types. For example, fishes are an important source of PUFA, especially those from ω 3 family, such as eicosapentaenoic (EPA), 20:5n3, and docosahexaenoic (DHA), 22:6n3. These classes of FA are very important to the biological systems and are EFA for humans. Olive oil, on the other hand, has a higher percentage of monounsaturated FA (around 70 %), mainly oleic acid, and a smaller proportion of PUFA (approximately 10 %). (117)

Dietary FA and their sources are discussed in the sections concerning the different FA classes.

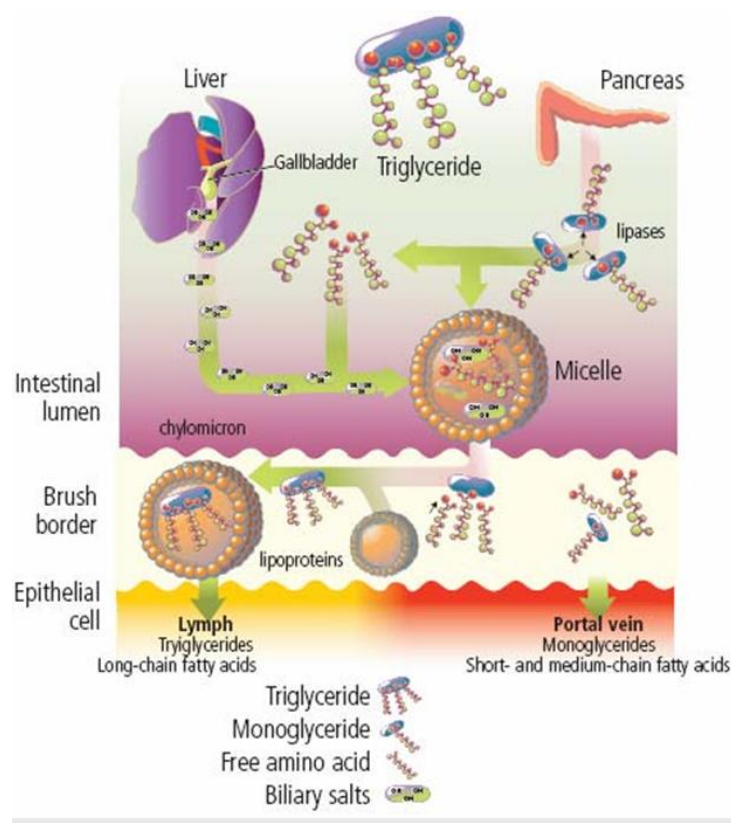
3.7.1. Digestion of fatty acids

Fatty acids are usually ingested in the form of TG, PL or CE. After ingestion, the dietary lipids suffer the action of the pancreatic lipase, in the initial part of the small intestine. Pancreatic lipase can only act in the oil/water interface; therefore, the presence of bile

salts to emulsionate the dietary fats is important, allowing a larger surface area for the enzyme to act. (91-92)

After digestion, FA of smaller dimensions are absorbed directly and fall in the venous portal system, entering the liver where they might be used as energy source, stored or used in the synthesis of lipoproteins. FA with longer chains are absorbed through the enterocyte apical membranes. Inside the enterocyte these FA are re-esterified into TG and PL and packed together to form chylomicrons (Figure 34). Chylomicrons are transient lipoproteins of very low density that appear in the circulation, mainly after meals. Chylomicrons are released by exocytosis in the intestinal lymphatic system capillaries – lacteals. (91-92)

Figure 34. Digestion and absorption of triglycerides.



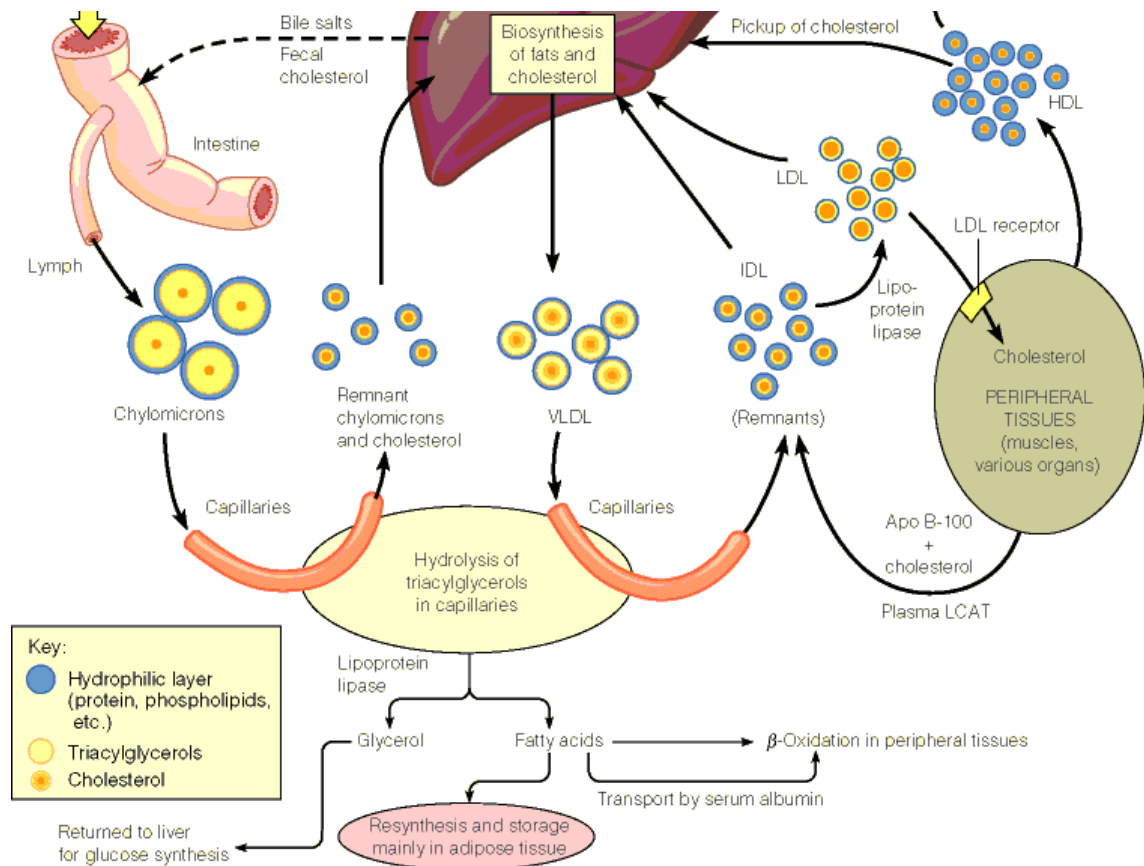
Adapted from internet. (118)

Chylomicrons leave the lymphatic system and enter the circulation in the subclavian vein. Skeletal muscle, adipocytes and hepatocytes can capture chylomicrons from circulation, to be used for energy or to be stored. (91-92)

Much of the chylomicron absorbed by the liver is used to synthesize lipoproteins of very-low density (VLDL). These particles, VLDL, are released in the circulation and transport TG across the body to different tissues. In those tissues lipase can hydrolyze TG present in VLDL. VLDL receive cholesterol from circulating lipoproteins and also modify their apolipoprotein profile, giving birth to a denser particle known as low-density lipoprotein (LDL). (91-92)

LDL, richer in cholesterol than VLDL, transport this important molecule across the body to tissues where it is needed (e.g. tissues that synthesizes steroidal hormones, as supra-adrenal glands). LDL presents apolipoprotein (apo) B100 that is recognized by LDL receptors and allows LDL to enter the cell. (91-92)

The high-density lipoprotein (HDL) is responsible for the transport of cholesterol back from the tissues to the liver, where it can be eliminated, used to synthesize bile salts or employed in the production of new lipoproteins. Figure 35 resume the lipoproteins functions as lipid transporters. (91-92)

Figure 35. Plasmatic lipoprotein and their lipid transport role.

Adapted from internet. (119)

3.8. Types of fatty acids

3.8.1. Free fatty acids

Free Fatty acids (FFA) is the name given to FA that are not linked to another molecule. In this state they are almost insoluble in water, thus, in circulation, they bind to albumin increasing their solubility almost 1000 fold, from 1μM to 1mM. Therefore, the amount of FFA in the circulation depends on the albumin available sites. (91-92)

3.8.2. Saturated fatty acids

A SFA present only single bonds in its carbon chain. A huge variety of saturated FA can be found in nature, ranging from the three carbon propionic acid to FA as long as the hexatriacontanoic acid, with 36 carbons. (88)

3.8.2.1. Health effects

Saturated FA are, usually, considered as unhealthy and are associated with increased risk for CVD. Some studies have found a decrease in CVD risk when saturated FA were substituted by PUFA and MUFA (120-122), while other studies reported no association between SFA and CVD. (123-124) The substitution of the energy coming from carbohydrates by SFA has been studied, but the results are not conclusive. (125) Palmitic FA has been associated with the development of CVD. (126)

A high intake of SFA is pointed to increase total cholesterol levels. (127) This effect is thought to be, mainly, by increasing the levels of LDL, usually without impact in HDL levels. (128)

Some types of cancer as breast, prostate and intestine cancer, have also been associated with the consumption of SFA. (129-131) Myristic and palmitic FA were particularly associated with prostate cancer. (131)

3.8.2.2. Food sources

Saturated FA can be found in different food products namely, butyric (4:0) acid in butter, lauric (12:0) and myristic (14:0) acids in dairy and some vegetal oils (coconut, e.g.), palmitic (16:0) and stearic (18:0) acids in meat, eggs and chocolate. (117)

Vegetal tropical oils, as coconut and palm oils, are important sources of SFA. (117) Due to the hot temperature in this zone of the globe, higher melting point of the SFA is not an issue to plants' physiology. (132)

3.8.2.3. Dietary needs

Studies have pointed that no minimum intake should be stated for SFA pointing that the human body can synthesize all the SFA it needs. (133) The American Heart Association suggests that the percentage of SFA should be under 7% of the total calorie intake. (134)

3.8.3. Monounsaturated fatty acids

MUFA present only one double bond in the carbon chain. Because of this single double bond MUFA present some physico-chemical characteristics between the SFA and the PUFA. For example, they are usually liquid at room temperature and present a melting point higher than PUFA and lower the SFA. (132)

The most important MUFA in human diet are the palmitoleic acid (16:1n7), the oleic acid (18:1n9) and the *cis*-vaccenic acid (18:1n7). *Cis*-vaccenic acid can be formed by the enzyme elongase adding two carbon units to the palmitoleic acid. (91-92)

3.8.3.1. Health effects

The intake of MUFA has not been linked to the development of coronary atherosclerosis, as occurs with PUFA. A possible explanation is the higher predisposition of PUFA for LP and, therefore, accumulation in arteries. (135-136) MUFA have been associated to a reduction in LDL and enhancement HDL. (147) In fact, children consumption of MUFA was associated with an improvement of lipid profile. (137)

The role of MUFA in IR is still to be established. Some studies reported that MUFA, together with SFA, increase the IR while PUFA reduces IR (138); others studies found no relation between MUFA and PUFA with IR, while SFA had a negative effect on it. (139) There are even studies that present MUFA as preventive in the development of IR. (140)

An increased risk of breast cancer, with increased levels of MUFA and reduced unsaturation index (UI) (calculated by the sum of the percentage of all the FA multiplied by their number of double bonds) in RBC membranes has been reported. (141)

Resuming, MUFA are generally associated with a better lipid profile, nevertheless its role on IR and cancer is still to be established.

3.8.3.2. Foods sources

Foods sources of MUFA are varied. Vegetal oils, as corn oil, canola oil, and sunflower oil, usually are good sources. In the Mediterranean diet, olive oil, with around 75 % of MUFA, represents an important source. (142)

Dairy products and milk have 30 % of MUFA in total fat. Meats from pork and beef present 40 % of fat in the form of MUFA; chicken and turkey present a lower amount of 20 %. (117, 142)

Nuts, cashew (around 60 %), almonds (65 %) and peanuts (50 %) are also a good source of MUFA. (117, 142)

3.8.3.3. Daily needs

The recommended amount of energy coming from fat is usually pointed to be 25 %. Because MUFA impact in health is still not well established, there is still some variability on the recommended daily intake. The values used proposed is that 10-15% of daily energy come from this type of fat (around 35-50% of the energy coming from fat). In fact, the daily intake of MUFA and PUFA should be similar. (142-143)

3.8.4. Polyunsaturated fatty acids

PUFA present more than one double bond in their carbon chains. These double bonds, usually of the *cis* type, introduce a curvature in the FA carbon chain, which gives to PUFA interesting physico-chemical properties, such as a decrease in inter-molecular interactions that avoid close packing at low temperatures. These lower inter-molecular interactions, ultimately, low the melting point of PUFA, to values that are lower than the other types of FA and will influence their role in biological systems. (83-84)

In fact, PUFA have an important role in animal and vegetal metabolisms. Two families are of particular importance, the ω 3 and ω 6 FA families, that despite their importance in the human biology, cannot be synthesized by mammals and, thus, have to be obtained from diet. (91-92)

Polyunsaturated FA are discussed in the sections corresponding to ω 3 and ω 6 FA.

3.8.5. *Trans* fatty acids

Trans FA are mono or polyunsaturated FA that present one or more double bonds in the *trans* configuration: the two parts of the chain, before and after the double bond, are in opposite sides. In result of this, instead of presenting a kink in the chain, like *cis* FA, *trans* FA chain is practically straight. This confers the molecule physico-chemical characteristics different from the *cis* isomers and somehow closer to the saturated FA. For example, oleic acid (18:1n9c) has a melting temperature of 13.4 °C, while elaidic acid (18:1n9t), an oleic *trans* isomer, have a melting temperature of 47°C. *Trans* FA can pack closer than the *cis* FA, thus, there is an increase of interaction between chains, leading to a higher melting temperature, when compared with *cis* FA. (144)

The majority of the FA existing in nature are not *trans*, but, in meat and dairy of ruminants, due to microbiological activity on their rumen, small amounts of *trans* FA are present, such as vaccenic acid (18:1n7t) and conjugated linoleic acid (CLA) (*cis*Δ9,*trans*Δ11-18:2n7). CLA presents two conjugated double bonds at both *trans* and *cis* configuration. The vaccenic acid can be metabolized by humans into CLA. (91-92)

The biggest part of *trans* FA consumption in western diets is product of industry processing of vegetal oils, especially from a process called hydrogenation. Hydrogenation is used to introduce hydrogen atoms in unsaturated FA double bonds in order to transform them in single bonds. (132)

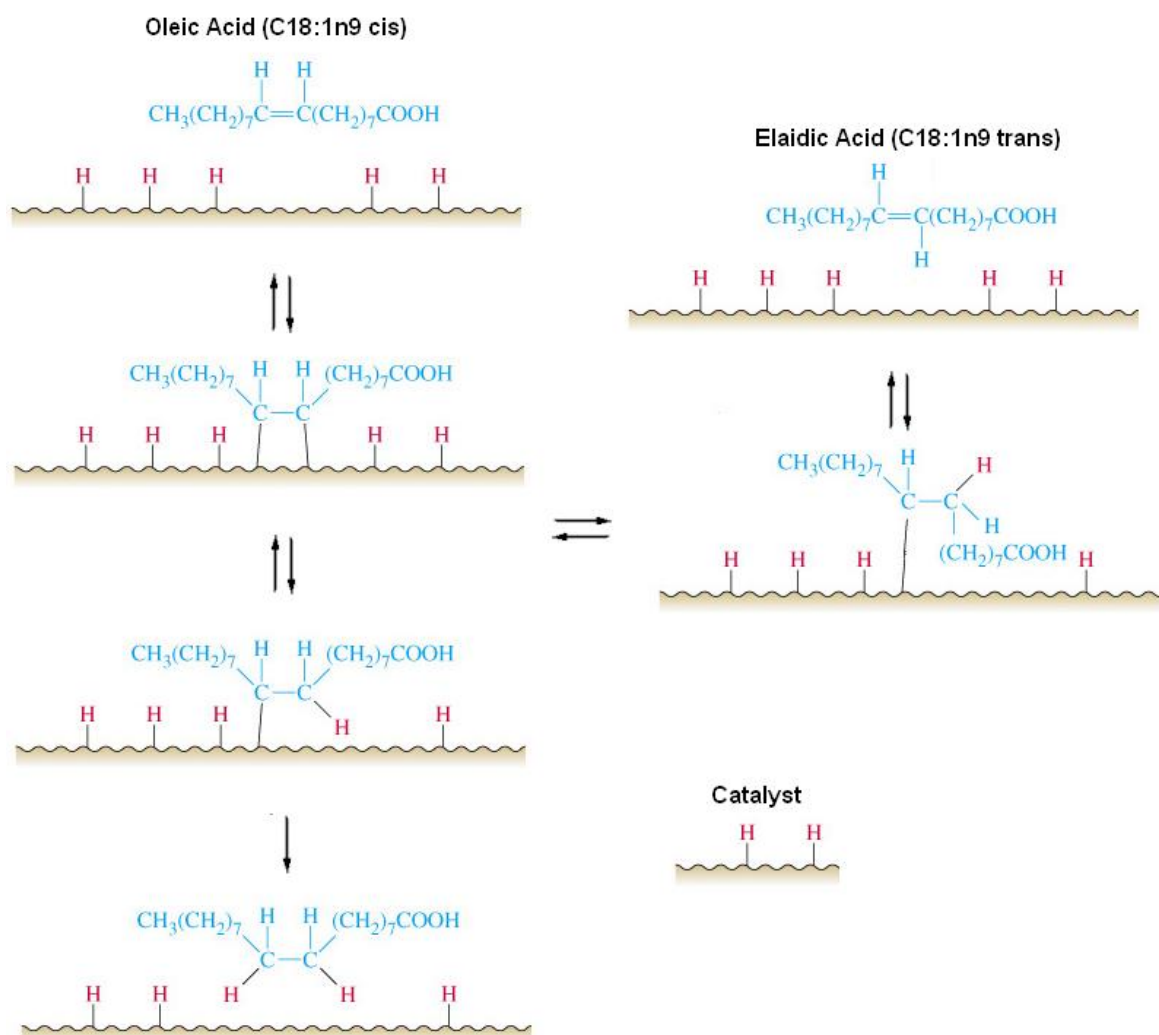
3.8.5.1. Hydrogenation of fatty acids

Hydrogenation can be called total or partial. In total hydrogenation, all the doubles bonds present are eliminated and only saturated FA are present in the final product; in partial hydrogenation, only part of the double bonds in the FA are transformed in simple bonds. The *trans* FA appear as a consequence of partial hydrogenation. (132)

During the catalyzed process of hydrogenation the first hydrogen is added to a carbon of the double bond, while the other is attached to the catalyzer. In the second step hydrogen is added to the other carbon of the double bond and the process finishes. However, the first step is reversible, meaning that the first hydrogen added can be released again. The

intermediate, with only one hydrogen, can rotate freely and if the double bond is reestablished, the configuration may “freeze” in *cis* or in *trans*. The *trans* configuration is energetically more favorable and, consequently, its percentage is usually higher (Figure 36). Traditionally, the *trans* FA may represent around 40% weight / weight of the final product of hydrogenation in the case of baking shortenings and around 15 % in margarines. (145-146) Products prepared with ingredients rich in *trans* FA (snacks, cookies, pre-fried food and fast-food) may present a percentage of around 45 % of the total fat being of *trans* FA. (145-146)

Figure 36. Hydrogenation of *cis* unsaturated fatty acid with the production of a *trans* isomer as a side product.



Adapted from internet. (147)

Hydrogenation is used by many reasons in industry: (132)

- To obtain solid products from vegetal oils;
- The products are of higher physico-chemical stability – More resistant to rancidity;
- It is a cheaper option to animal fat and to semi solid vegetal oils (Palm oil, e.g.);
- Products present a longer shelf life and smaller refrigeration needs.

Partial hydrogenation is more used than total hydrogenation, because the product obtained is solid at room temperature, but still melts when necessary (e.g. baking or spreading margarine in bread). (132)

Many governments limit or prohibit *trans* fat. One of the solutions that some producers found to lower the *trans* fat content of their products is to modify the conditions of hydrogenation (temperature, pressure, time). Another approach is by adding vegetable oils to the hydrogenation products, thus lowering the relative percentage. (148)

3.8.5.2. Health effects

Trans FA have been associated with CVD. (149) In fact, like SFA they are thought to increase LDL levels. Besides that, *trans* FA are associated with a reduction of HDL. Therefore, the traditional ratio LDL/HDL, used as a measure of risk for CHD, increases to almost the double with the consumption of *trans* FA when compared with SFA. (128) The levels of C-reactive protein (CRP) seem to increase with the consumption of by *trans* FA. (132)

Trans FA relation with atherosclerosis, in a similar way to SFA, might be related to the fact that *trans* FA form more stable atherosclerosis plaques. Due to their molecular configuration, *trans* FA and SFA can interact closer with adjacent FA, leading to a stronger and more resistant plaque, even at the human body temperature. UFA lead to more malleable plaques. (132)

Besides CVD, the impact of *trans* FA in many diseases has been studied. An increased weight gain with the consumption of *trans* FA was described. In a study made with monkeys, *trans* FA increased the weight gain, the intra-abdominal deposition of fat, and decreased insulin sensitivity through impairing the insulin receptor signal transduction. (150) This relation of *trans* FA with IR have been the focus of studies relating *trans* FA

with diabetes. Some of these studies have a relation with increased *trans* FA intake and the risk of diabetes (151), while others have not. (152)

Cancer association with *trans* FA also has deserved attention. A direct relation between the consumption of *trans* FA and prostate (153) and breast cancer (154) have been pointed out. However, others studies have found no relation or even a decrease in cancer risk with the consumption of *trans* FA (lower incidence of aggressive prostatic cancer). (155)

Mood disorders have been associated with *trans* FA in the diet. An increase in depression with higher diet levels of *trans* FA have been described. (156)

Even when considering the amount of calories provided by FA in comparison with other nutrients, *trans* FA are those that increase more the risk of CVD. (149) Also, when compared to SFA, *trans* FA further increase the risk of CVD. The substitution of 2% of the energy provided by carbohydrates by *trans* FA increased in 2 fold the risk of CHD, it was necessary a substitution of 15 % of carbohydrates dietary energy by SFA to have a similar effect. On the other hand, the consumption of unsaturated *cis* FA instead of saturated and *trans* FA decreased the risk of coronary heart disease (CHD). (157)

The presence of *trans* FA seem to impair the activity of the enzyme delta 6 desaturase, an important enzyme in the synthesis of FA of the ω 3 and ω 6 family. (158)

In contrast, natural occurring *trans* like conjugated linoleic acid (CLA) and vaccenic FA appear to have positive health effects, by lowering LDL and TG. (159) The FDA definition of *trans* FA excludes these natural occurring conjugated *trans* FA by stating that *trans* FA are FA with one or more non conjugated double bonds in the *trans* configuration. By stating “non-conjugated” CLA is excluded from the definition. (160)

3.8.5.3. Food Sources

In western diets the intake of *trans* FA is, mainly, from processed oils and fats. An important natural source is meat and dairy of ruminants, although these FA are produced by microbiological activity on the animal rumen, and not by the animal. Natural occurring FA appear to have a beneficial health impact. (159)(159)(159)(152)¹⁰⁷

3.8.5.4. Dietary needs

There is no minimum intake for *trans* FA intake, because they are not necessary for the normal function of the body; they present no known benefits and have been associated with deleterious health effects. (149) Regarding the upper limit, the World Health Organization stands that not more than 1% of total energy should come from *trans* FA (161) while the American National Academy of Science states that there is no safe level for *trans* FA due to their harmful health effects. (142)

3.8.6. Essential fatty acids

When firstly discovered as essential to humans, EFA were named as vitamin F. (162)

There are two EFA:

- Linoleic acid (LA), a ω -6 FA with unsaturations on carbons Δ 9 and 12;
- Alpha-linolenic acid (ALA), a ω -3 FA with unsaturation on carbons Δ 9, 12 and 15.

They are essential because humans (and other mammals) do not possess desaturases able to introduce double bonds in carbons further than carbon 10, counting from the carboxylic end. (162)

Although the whole ω 3 and 6 families are sometimes called “essential”, humans can synthesize FA with longer chains or more unsaturations from both families parting from LA and ALA. For example, humans can synthesize arachidonic acid (AA), 20:4n6, from LA, and eicosapentaenoic acid (EPA) and docosapentaenoic acid (DHA) from ALA. Nevertheless, these metabolic processes to obtain FA by further elongating and desaturating is energy demanding, being simpler to the organism to obtain these FA from dietary fats. (162) In fact, the percentage of EPA and DHA from endogenous production is much smaller than that obtained from diet. Studies with tissue membranes, often consider that all of these FA are exogenous. Almost all the PUFA obtained from diet are from the families called “essential”. (163)

3.8.6.1. Biological role

Essential FA have important biological functions, namely: (91-92)

- They are the basis for the production of eicosanoids, endocannabinoids, ... Thus, they are involved in the modulation of inflammation,
- They affect platelet aggregation,
- They influence membrane fluidity and, through it, the function of membrane receptors, channels and cellular signaling,
- They influence gene expression.

The EFA are discussed separately in the sections of ω 3 and 6 FA.

3.8.7. Omega 3 fatty acids

Omega 3 FA present the first double bond 3 carbons before the ω carbon. The most relevant ω 3 FA in human biology are ALA (ALA – 18:3n3), EPA (20:5n3) and DHA (22:6n3); all their double bonds are in the *cis* configuration. (164)

The ALA is, in fact, the only essential ω 3 FA, as EPA and DHA can be synthesized from it. However, this conversion has an efficiency below 5% in man and a little higher in women. (163) Besides this low efficiency, the endogenous ω 3 pathway shares the same enzymes with the ω 6 synthetic pathway and, thus, have to compete for them. Consequently ω 3 FA have their synthesis diminished in the presence of high ω 6 concentrations. (165) The easiest way to increase the percentage of ω 3 in the tissues is, therefore, by increasing their consumption or limiting the ingestion of ω 6 FA. Controversially, there are researchers referring that it is the ω 3 absolute concentrations, and not its ratio with ω 6, that limits the conversion rate. (166)

In children omega 3 FA are essential for a normal growth and development (167) It has been shown that ω 3 containing formula might improve maturation of the immune system in infants. (168) The importance of ω 3 in the development is already important in the intra uterine fetal development. It was reported that the consumption of fish, an important source of ω 3, during pregnancy, reduced the risk of low birth weight and premature delivery. (169)

Omega 3 FA are important also in the development and maintenance of brain function. They fortify the myelin membrane sheets and promote neuron growth, consequently

helping the brain repair mechanisms. (170-171) DHA has an important role in the brain structure, especially in the grey matter, by allowing proper neurotransmission and retinal stimulation. (170)

The ω 3 FA seem to have also an important role in cardiovascular health, especially EPA and DHA. ALA has not been proved to have the same effects. (172-173)

DHA and EPA are present in high concentrations in cold water oily fish, as salmon and sardines, but are not synthesized by them. The original producers of these FA are microalgae. Their FA concentrations then accumulate up in the food chain. (157) PUFA are important in these cold water fishes because they help keeping the cellular membrane fluid in very low temperatures. (174)

3.8.7.1. Health effects

EPA and DHA have been associated with many health benefits such as CV protection and TG lowering properties. (175-176) EPA and DHA have been proposed to increase blood flow and to decrease clot formation, blood pressure (177-178), the risk for primary and secondary heart attack (173) (39), cardiac arrhythmias (179), and the risk for thrombotic and ischemic stroke. (49) Thus, fish and fish oil supplements seem to be reduce cardiovascular events and their associated mortality, as they are rich in ω 3 FA. (180) Some of these cardiac effects might be related with the fact that ω 3 FA may increase the fibrillation threshold of the heart tissue and, thus, prevent some of the cardiac events, especially those related with arrhythmia. (181) However, some other studies showed no relation between ω 3 FA and cardiovascular protection. (171)

A reduction in the carotid artery thickness with the consume of EPA in T2DM patients was described. (182) Also a reduction of TG and VLDL was observed in individuals under EPA and DHA supplementation (4 grams / day). (183) EPA also reduced the circulating antibodies anti-oxidized LDL, which are a well known mediators in the development of atherosclerosis. (184)

A beneficial effect is also attributed to ω 3 FA in inflammatory diseases, presenting these FA an anti-inflammatory activity. (185) A positive effect was also found in rheumatoid arthritis. (186-187)

Of great interest was also the study of the effects of EPA and DHA in mood and psychiatric disorders. There were positive results in depression (188), even in pediatric ages (189), and anxiety. (190) Also in bipolar disease EPA and DHA supplements helped stabilizing the mood. (191) These supplements can be of natural fish oils or synthetic like E-EPA (a synthetic EPA-ether). (192) However, other studies did not found a connection between ω 3 FA and the prevention of depression. (193) An interesting study showed that suicide attempt patients present a lower blood level of EPA. (194)

Children and adolescents with tendency to schizophrenia present lower levels of ω 3 (and ω 6) FA. A supplementation with DHA and EPA reduced the progression to the disease. (195) Due to their role in brain development and repair mechanisms, ω 3 synthetic EPA ester (E-EPA) supplementation increased the grey and white matter in the brain of individuals suffering from schizophrenia and Huntington's disease. (196) Children with attention deficit disorder also appear to benefit from ω 3 consumption. (197)

The impact of EPA and DHA in cancer has been studied and contradictory results have been found. Some studies found a beneficial effect in the development of breast, colon and prostate cancer. (141, 198-199) An improvement of weight and appetite in cancer patients has also been described with EPA and DHA supplementation. (141) Women presenting higher concentration of DHA in the RBC membranes present a lower risk of developing breast cancer. (141) On the other hand, some studies found no association between ω 3 FA and cancer (200) and others found a worsening of the risk for aggressive prostate cancer. (155)

3.8.7.2. Food sources

Fishes are the richest source of EPA and DHA FA, which are the ω 3 FA with more putative health benefits. Sardines, salmons, anchovies and other cold water oily fishes are the best sources. The ratio of ω 6 to ω 3 in these fishes is about 1:7. In fact, fishes do not synthesize these FA, they are produced by microalgae and plankton, present in these fishes diet. (157)

However, it is not only the ω 3 FA that accumulate up the food chain, dioxins and heavy metals are a well known problem of fish eating. However, the health benefits are pointed to outweigh the risks. (201)

Krill oil is also an option for a ω 3 EPA and DHA source and seems to have the same metabolic improvements at a lower dose of these FA. (202)

The majority of the vegetables do not have a high percentage of ω 3 FA. Their approximate ω 6 to ω 3 ratio is: Corn oil, 46:1; olive oil, 13:1; soy bean, 7:1; sun flower oil, cotton seed oil and peanut oil have approximately no ω 3. There are exceptions, as the flaxseed that presents a ratio of 1:3 and canola a 2:1 ratio. (203-204) Even the vegetables that present a higher percentage of ω 3 FA present mainly ALA, being the percentage of EPA and DHA too small to be a valid source of these two FA. (205)

Eggs of grain feed chicken have higher amounts of ω 3 FA than chicken, traditionally fed with corn. However, this ω 3 content is mainly of ALA. Adding flax seed to the chicken ration only increases their content in ALA. To increase the content of eggs in EPA and DHA it might be used algae in their diets; a problem with this approach is that the eggs can taste like fish. (206) Meat from animals grown freely and eating grass have higher amounts of ω 3 FA (almost 3 fold), but, once again, mostly in ALA. (207)

Consumers must pay attention to false labeling. Many of the foods supposed to be enriched with ω 3 FA are, actually, only enriched in ALA. ALA does not present the health potential of EPA and DHA and have to be metabolized by humans into those FA. (205)

3.8.7.3. Dietary needs

According to the US Institute of Medicine of the National Academy, the acceptable ω 3 intake is 1.6 gram / day for men and 1.1 grams / day for women, without setting an upper limit. (142) They should provide from 0.6 to 1.2 percent of total daily energy. (142)

The FDA stands that the Daily intake of ω 3 FA should not exceed 3 grams per day, with no more than two grams coming from supplements. (198, 208)

The American Heart Association has even proposed different amounts, according to health conditions and objectives:

- Healthy individuals should have 2 meals of oily fish per week;
- Individuals with CHD should eat 1 gram of DHA and EPA daily;
- Individuals with high TG should take 2 to 4 grams of EPA and DHA supplements daily.

Excess of EPA and DHA may have deleterious effects, such as: increased risk of hemorrhagic stroke (209), increased risk of death in congestive heart failure patients (141), increase of LDL and reduced glycemic control in diabetes patients. Supplementation of ω 3 FA for patients in these risk groups should be discussed with a clinician.

3.8.8. Omega 6 fatty acids

Omega 6 FA present the first double bond 6 carbons away from the ω carbon. The physiological most important FA in this class are LA (18:2n6) and AA (20:4n6). The interest by the scientific community in the γ -linolenic acid (GLA - 18:3n6) and its derivate dihomo- γ -linolenic acid (DGLA - 20:3n6) has been growing lately. (132)

The biological actions of ω 6 FA are in great part related to the production of eicosanoids. Eicosanoids are substances synthesized from 20 carbons FA that mediate many functions in the body as inflammation, tissue repair and clotting. (210)

Omega 6 FA compete with ω 3 for the same enzymatic pathways and also for the presence in biological lipids, as in membrane PL and TG. (210-212)

3.8.8.1. Health effects

High levels of ω 6 FA in the diet have been associated with diseases. (213) Although the optimal ratio between ω 6 and ω 3 FA should be around 4:1 or even lower, the modern diets offer ratios of around 10:1 or even 30:1. (214)

Omega 6 and omega 3 FA compete for the same enzymes, either for the synthetic pathway or for the eicosanoid metabolism. When the levels of ω 6 FA increase, the metabolic pathways turn towards the increase of inflammation, thrombosis and hypertension. (215) (86) They are also associated with many diseases, most of them with an inflammatory basis: obesity, arthritis, asthma, cancer and CVD. (211) Controversially, some argue that the consume of ω 6 FA may reduce the risk for developing CVD. (210)

Increased ω 6 FA in diets have been related with breast (216) and prostate (217) cancers. Actually, ω 6 are a class of FA and not all FA from this class present the same health effects. In fact, DGLA has been pointed to present anti-cancer effects. (141)

The treatment of some diseases use the inhibition of the enzymes related with eicosanoids synthesis: inhibition of COX 2 in arthritis (218) and of LOX in asthma; the inhibition of AA cascade can also be used to prevent mania symptoms (219); increasing the intake of ω 3 FA, and, thus, dislocating ω 6 FA from the metabolic pathway can also be used. (220)

3.8.8.2. Food sources

The food sources for ω 6 FA are, typically, vegetable oils, specially, soybean, sunflower and palm oil. (221) Also cereals, nuts and whole grains are important sources of ω 6 FA. (117)

Eggs and meat from animals grown in intensive farms, usually, have higher proportions of ω 6 to ω 3, when compared to animals grown freely. (206, 222)

3.8.8.3. Dietary needs

A very interesting report article by Cunnane (223) pointed that the dietary needs for ω 6 FA might be overestimated, since the animal experiments to determine these amounts were done using ω 6 and ω 3 deficient diets. In this way, the concomitant ω 3 deficiency might have caused an increase of the ω 6 demand by 5 to 15 fold. Since our body needs more ω 3 than ω 6 FA, the tested diet, deficient in both types of FA, accentuated the deficit's symptomatology, that was considered to come only from the ω 6 FA deficiency. (223)

3.8.9. Omega 9 fatty acids

Omega 9 FA present an unsaturation at the 9th carbon away from the omega carbon. (88) These FA are not essential for humans, as they can be synthesized from saturated FA. (91-92)

Most of the relevant ω 9 are monounsaturated FA, as the oleic (18:1n9), the eicosaenoic (20:1n9), the erucic (22:1n9) and the nervonic (24:1n9) acids. The 5, 8, 11-eicosatrienoic acid, or mead acid, is one of the exceptions; it is formed in case of starvation or unbalanced vegan diet. In these cases the mead acid is synthesized by the organism from saturated or oleic acid, in order to minimize the deficit in ω 3 and 6 PUFA, as LA, ALA, EPA and AA acids. (224-225)

Omega 9 FA cannot participate in the eicosanoid synthesis because, they do not have a double bond in n6. They are generally considered anti-inflammatory. (210-212)

Oleic acid is found in many food sources as vegetal oils, specially olive oil, animal fats, and nuts. Erucic acid is present in canola oil. (117)

The food sources and health impacts of ω 9 FA are discussed more deeply in the MUFA section.

3.9. Fatty acids and inflammation

3.9.1. Omega 6 / Omega 3 ratio

Many studies evaluated the importance of balancing the ingestion of ω 6 and ω 3 FA. The control of this ratio appears as a key point to prevent CVD. (226) (34)

It has been pointed that a healthy ω 6 / ω 3 ratio would range from 1:1 to 4:1. (215, 227) Traditional human diets used to match these ratios, nowadays, however, this value is, usually, close to 10:1 or, sometimes, to 30:1. (228) This changes in the modern diets, apart from the traditional diet, may lead to many of the health problems find nowadays. (229)

By lowering this ratio (through increasing ω 3 and lowering ω 6), it is possible to lower some of the pro-inflammatory ω 6 mediated effects, and to change the eicosanoids synthesis towards the less inflammatory ω 3 products. Besides, there are other positives effects of ω 3 on the organism. (204, 230)

Omega 3 (and 6) FA may also modulate the immune function by modifying the cellular membrane and it's lipid rafts constitution and, consequently, modulate the action of membrane receptors and channels, also, by modulating gene transcription, they can interfere in cytokines synthesis, for example. (191)

To some authors, however, the absolute values of ω 3 ingestion are the main determinants for the prevention of CVD, having ω 6 little or no effect. (210)

3.9.2. Eicosanoids

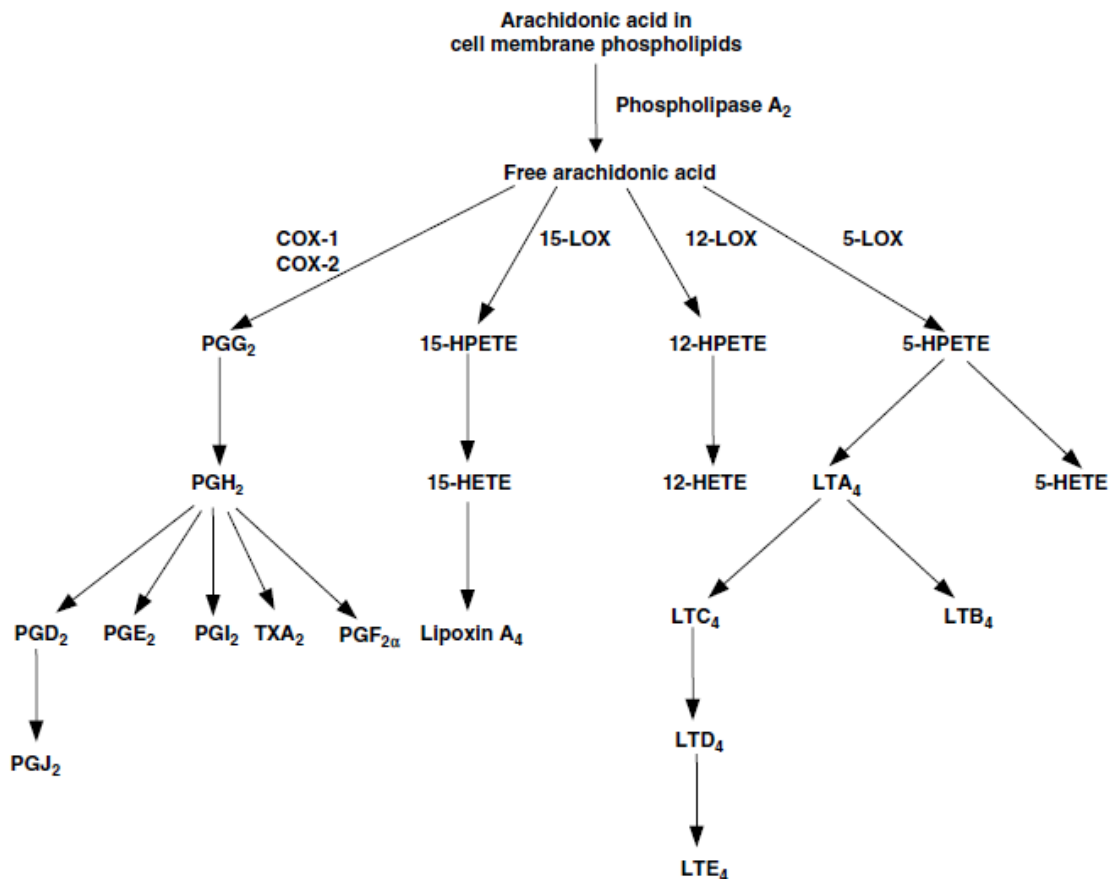
Eicosanoids are the name given to a class of molecules derived from the metabolism of polyunsaturated FA with 20 carbons - ω 3, EPA, and ω 6, AA and DGLA. There are 4 classes of classical eicosanoids: prostaciclins (PGI), prostaglandin (PG), thromboxanes (TX) and leucotriens (LT). PGI, PG and TX are synthesized by cyclooxygenase (COX), while LT are originated by the action of the enzyme lipoxygenase (LOX) (Figure 37). (210-212)

To the eicosanoids synthesized by COX (PGI, PG and TX) are named prostanoids. Each class of eicosanoid is divided in series of related molecules. There are three or two series per class. (101, 204)

Lately, other types of molecules have been studied that are technically eicosanoids, like endocannabinoids, resolvins, isofurans and epoxyeicosatrienoic acids. These molecules are called novel or non-classical eicosanoids and appear to have important functions, especially, in the central nervous system (CNS). (169, 231)

The classic eicosanoids are, usually, presented with their abbreviated name. This name is formed by the class abbreviation (PGI, PG and TX), followed by a letter that identifies the series, and a number referring to the number of double bonds. Stereochemistry is described by Greek letters (e.g. PGF 2α). Eicosanoids with different letters present different functional groups and double bonds in different carbons. Thus, the TXA $_3$ is the TX from the A series and with 3 double bonds (originated from the EPA metabolism). (101, 204)

Figure 37. Eicosanoids synthesized from arachidonic acid.

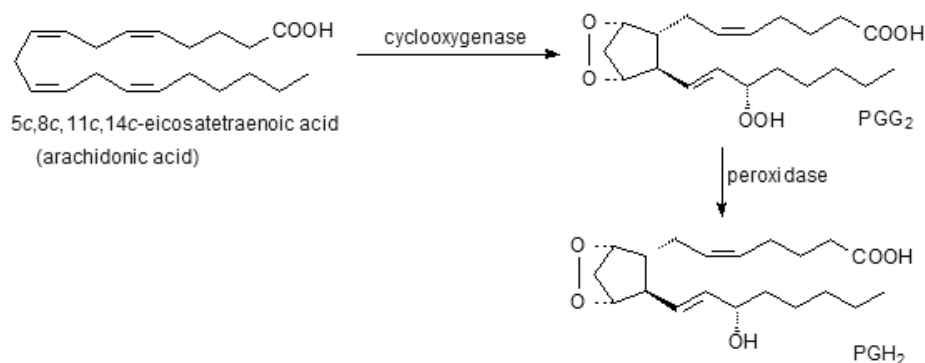
Adapted from Calder *et al.* (212)

Eicosanoids do not accumulate in cells, as a pre-formed reserve. They are produced in response to a stimulus that may be another eicosanoid, a cytokine or a mechanical stress that trigger the beginning of eicosanoid synthesis. Those stimuli cause a phospholipase to be released from the cytoplasmic membrane – phospholipase A₂ (PLPA₂). The cytoplasmic PLPA₂ leads to the release of a 20 carbon FA from a PL in (Figure 37). The action of PLPA₂ is the limiting step of the eicosanoid synthesis. Afterwards, these FA are metabolized to form the eicosanoids, through the enzymes COX and LOX. (101, 145, 204) PLPA₂ is specific for PL with 20 carbons FA on the position sn2. (101)

The enzyme COX removes two double bonds from the FA through reaction with oxygen. In this way, by the action of COX, EPA will generate prostanoids with 3 double bonds, AA will be transformed in 2 double bond prostanoids and DGLA will originate prostanoids with only one double bond (Figure 38). (101, 204) COX also presents two isoforms, the

constitutive COX-1, related with stomach mucosa protection and correct hemostasis, and inductive COX-2, that synthesizes prostanoids in reaction to certain stimulus. (210-212)

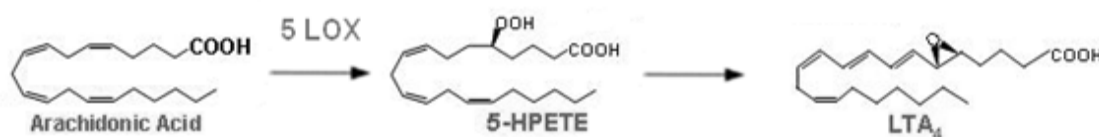
Figure 38. Synthesis of PGG₂ and PGH₂ from arachidonic acid by cyclooxygenase and peroxidase.



Adapted from internet. (232)

On the other hand, LOX reaction with oxygen is not done through the double bond interaction, thus, it does not eliminate double bonds (Figure 39). The LT originated possesses the same number of double bonds as the original molecule. There are different types of LOX named according to the position in the carbon chain to which the oxygen is added. The 5-LOX is the LOX responsible for LT synthesis. (101, 204)

Figure 39. Synthesis of 5-HPETE and LTA₄ from arachidonic acid by 5 lipooxygenase.



Adapted from internet. (233)

The action of COX and LOX leads to the formation of ROS intermediates (as lipoperoxides). Besides, that the eicosanoids are themselves highly reactive particles that

can damage DNA and other macromolecules. Thus, the synthesis of eicosanoids has to be tightly controlled, to avoid side damages. (101, 204)

Eicosanoids usually have a short lifetime in the organism, ranging from seconds to minutes. After synthesis they are rapidly metabolized and inactivated. In pathological conditions the balance between synthesis and catabolism of eicosanoids is broken and deleterious effects may appear. EPA and DGLA compete for the enzymes involved in the eicosanoid synthesis. Their products are considered to be less inflammatory than AA. (234)

Each eicosanoid presents a particular action that is mediated through membrane receptors or by acting directly in the nucleus, modulating DNA transcription. Eicosanoids act through receptors autocrinally and paracrinally. The effects of eicosanoids are mediated through receptors that are mostly members of the G protein coupled family. The same eicosanoid can bind to different receptors and, through these receptors, cause different effects, ranging from anti-inflammatory to pro-inflammatory effects. The same cell may present different eicosanoid receptors with opposing functions. (235) The FA that originate eicosanoid can also diffuse to the nucleus and modulate the DNA transcription of inflammatory mediators. (101, 236)

Prostanoids are proposed to increase inflammation. For example, they are responsible for inflammatory symptoms like edema, swelling, pro-coagulation state, pain and fever. (237) Leukotriens have an important role in inflammatory diseases, such as asthma and arthritis. They are also involved in immediate hypersensitivity reactions. This biological effect is due to leukocyte chemotaxis for LT and to the release of enzymes and ROS produced by neutrophils, they may also cause bronchoconstriction and do other actions. (231)

The role of AA, EPA and DGLA in the brain is different than in other tissues. Stimulus by neurotransmitters would activate the phospholipase that would free the FA. The free FA, under the action of specific enzymes, could be transformed in eicosanoids, endocannabinoids and neuroprotectin. These compounds can act in the same cell or paracrinally, modulating pre and post synaptic transmission, the release of neurotransmitters or even altering DNA transcription inside the nucleus. (238)

3.9.3. Omega 3 and omega 6 balance and eicosanoid synthesis

The ω 3 and ω 6 FA compete for the same pathways to generate the eicosanoids, producing similar products that, however, may present opposite effects. In this way, the ratio of ω 6 to ω 3 will influence the eicosanoids that are produced. Consequently, the dietary balance between these two FA families will modulate the eicosanoids production. (234) Counter-acting the pro-inflammatory effect of AA is probably an important reason for the ω 3 beneficial impacts. (239)

The ω 3 FA from diet, especially EPA, and the ω 6 DGLA may counteract the AA's eicosanoids synthesis in three ways:

- Displacement: the dietary ω 3 FA decrease the accumulation of AA in the tissues, lowering its availability to be used in eicosanoid synthesis. ALA prevents the LA elongation and desaturation to form AA, while EPA directly decreases the membrane concentrations of AA. (240)
- Competitive inhibition: the presence of higher concentrations of EPA and DGLA will dislocate AA from COX and LOX actions, thus, reducing AA related eicosanoids. (236)
- Counteraction: The eicosanoids products of EPA and DGLA directly oppose the effects of AA's eicosanoids in tissues, by presenting either a less inflammatory or an anti-inflammatory effect. (101, 236)

DGLA may result from LA, preventing the formation of healthier ω 3 FA. The best way to increase the levels of DGLA is GLA supplementation or the consumption of GLA rich foods. In this way, the impact of synthesized DGLA is smaller on the ω 3 synthesis. DGLA may be a precursor of AA acid, and, therefore, it would be expected that following GLA supplementation, the levels of AA and related inflammation would rise, but this does not seem to happen. (241)

Synthetic FA ester, as E-EPA, may be used as supplements to change the balance towards ω 3 derived eicosanoids. (166) Dietary anti-oxidants may also interfere in the eicosanoid metabolism. For example, red wine *trans*-resveratrol seems to reduce the TXA₂ synthesis. (242)

Docosanoids

In a similar way to 20 carbon FA originating eicosanoids, 22 carbons FA, especially ω 3 DHA, provides the basis for the synthesis of bioactive substances by oxygenation – the docosanoids. The biological action of DHA must be due in part due to these molecules. (231, 243-244)

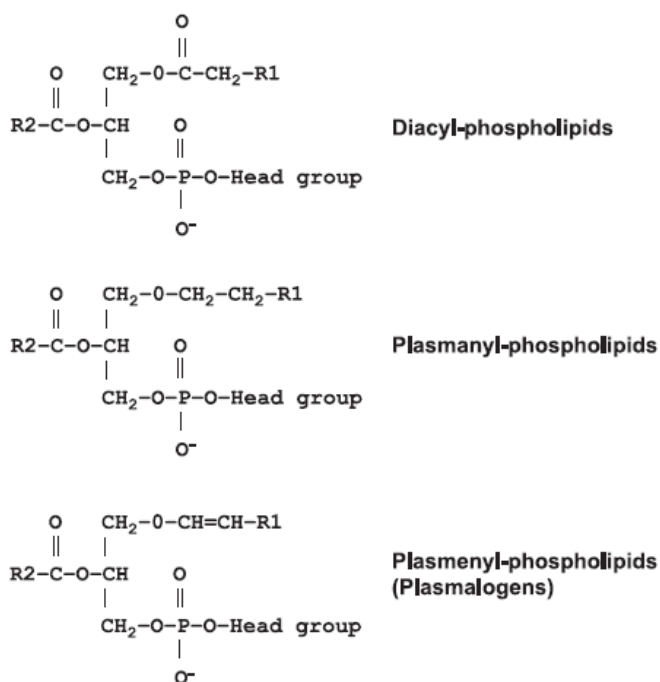
Resolvins, docosatrienes, neurofurans are some examples of docosanoids. Docosanoids appear to have important actions in the CNS functions. Neuroprotectins, for example, are a class of docosanoids that are anti-inflammatory and neuroprotective (as the name indicates). (231, 243-244)

4. Plasmalogens

Plasmalogens are part of the ether-PL group, which is characterized by the presence of an ether bond in the sn1 position instead of the ester bond in the traditional glycerol phospholipids. There are two types of ether PL:

- Plasmanyl-PL – Present an ether bond in the sn1 position, e.g. the platelet activating factor.
- Plasmenyl-PL – Have a vinyl-ether bond in the sn1 position, e.g. Plasmalogens (245-246) (Figure 40).

Figure 40. Plasmalogens structure in comparison to glycerophospholipid and plasmanyl-phospholipids.



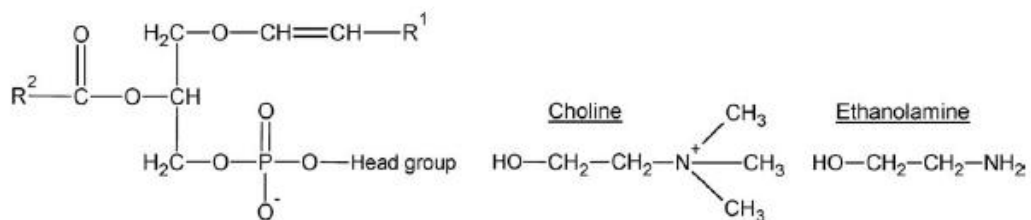
Adapted from Brites *et al.* (245)

The vinyl-ether group is responsible for some of the biological plasmalogens functions. Their structure constitutes of a glycerol backbone with a fatty alcohol bound by a vinyl-ether bond to the sn1 position, usually palmitic, stearic or oleic acids derived fatty alcohols; a fatty acid bound to the sn2 position linked by an ester bond, usually ω 3 and ω

6 PUFAs; and a polar head group bound to the phosphate, choline or ethanolamine. (245-246)

The head group composition will define two classes of plasmalogens: glycerophosphoryl-ethanolamine plasmalogens (PlsE) and glycerophosphoryl-choline plasmalogens (PlsC) (Figure 41). PlsE presents, usually, in the sn2 position with AA or DHA while PlsC circulates mainly with 34, 36 or 38 carbon chain species (saturated and unsaturated). (245-246)

Figure 41. Plasmalogen head groups.

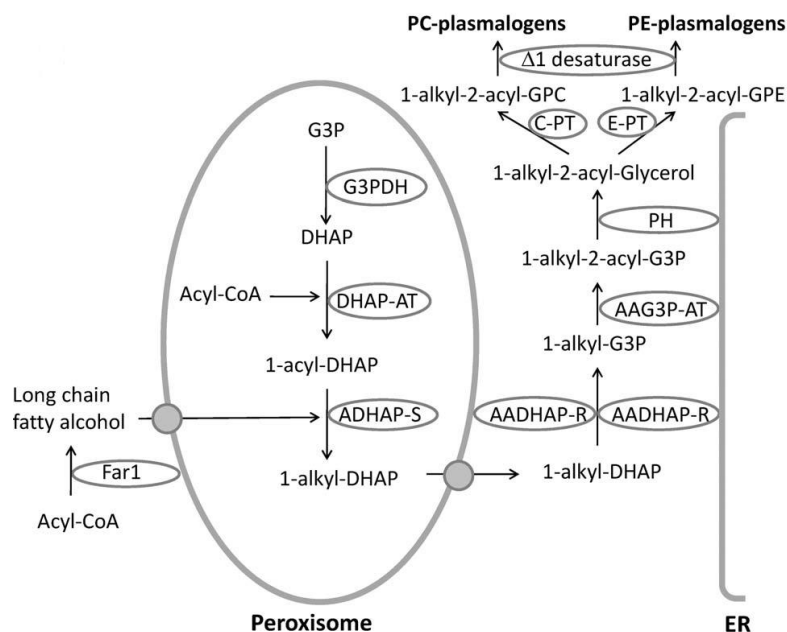


Adapted from Wallner *et al.* (246)

4.1. Synthesis

Plasmalogens are mainly synthesized in the liver and circulate bound to plasmatic proteins. Actually, they have been pointed to act as anti-oxidants of plasmatic lipoproteins. The half-life of plasmalogens is very short - 30 min for PlsC and 3 hours for PlsE. (245-246)

Plasmalogen synthesis begins in peroxisomes, the first two steps, and proceeds in the ER. The peroxissomal steps are critical for plasmalogens synthesis. Indeed, diseases characterized by loss or diminished peroxissomal function present, typically, reduced plasmalogens tissue levels with serious deleterious effects, especially, in the CNS functions. The fatty alcohol used for sn1 position is usually synthesized from the corresponding FA; alternatively, it can come from the diet. Figure 42 resumes the plasmalogens synthesis. (245-246)

Figure 42. Synthetic pathway of plasmalogens.

DHAP-AT, dihydroxyacetone-phosphate acyltransferase; ADHAP-S, alkyl-dihydroxyacetone phosphate synthase; Far1, fatty acyl CoA reductase 1; AADHAP-R, acyl/alkyldihydroxyacetone phosphate reductase; AAG3P-AT, alkyl/acyl-glycero-3-phosphate acyltransferase; PH, phosphohydrolase; E-PT, ethanolamine-phosphotransferase; C-PT; choline-phosphotransferase; ER, Endoplasmatic reticulum. (246)

Plasmalogen synthesis decreases with age. In neonates plasmalogens pathway may not be totally developed as their concentration in RBCs is smaller than in children, this way lead to higher susceptibility to oxidative stress in neonates. (247)

4.2. Distribution in tissues

Plasmalogens are present in several mammalian tissues representing a 18 % of PL mass in humans. This value may change for the different tissues; in CNS is much higher – 30-50% of ethanolamine glycopospholipids in the brain are plasmalogens. In fact, plasmalogens appear to have a very important function in the correct development of CNS, being involved in the formation of myelin sheaths and correct neuron migration. Granulocytes are the blood cells that present a higher plasmalogen concentration in the plasmatic membrane, while the RBC presents the lowest. (245-246, 248)

Besides, the change in total plasmalogen concentration between tissues, the two classes of plasmalogens, PlsE and PLC, have also different proportions and, thus, confer different

functions. In the heart there is more PlsC - 16% vs. 15 % of PlsE. Contrarily, in the brain there is about 25 times more PlsE than PlsC (22.5 % vs. 1%, respectively); in other tissues, as kidneys, lung and testis, the ratio PlsE:PlsC is 10:1. Differences between different tissues are not only present in plasmalogens classes, but also in the alkyl and acyl substituents in sn1 and sn2 position, respectively. For example, in the CNS, the white matter presents a higher percentage of MUFA in the sn2, allowing a closer interaction between the FA chains and, thus, forming a more compact myelin sheath. On the other hand, the grey matter has higher percentage of PUFA in the sn2 position. PUFA increase membrane fluidity and facilitate membrane fusion and the communication process required by neurons. (245-246)

Plasma lipoproteins present different plasmalogens concentrations as well. The HDL fraction contains around 60% of lipoprotein PlsE. The different lipoproteins present different plasmalogens profiles, with HDL and VLDL presenting more long chain saturated species, while LDL have a higher concentration of monounsaturated plasmalogens. HDL also presents the higher proportion of arachidonic acid in the sn2 position. (245-246)

4.3. Membrane function

Plasmalogens are important in membrane functions. They increase membrane fluidity, due to the high percentage of PUFAs in the sn2 position. The vinyl ether group allows plasmalogens to form an inverse hexagonal phase that is crucial for membrane fusion events, particularly in CNS neurons. (245-246)

The cells presenting low levels of plasmalogens have lower levels of cholesterol esterification and, consequently, diminished intra and extra-cellular cholesterol transport and increased free cholesterol in the membrane. Besides that, these cells also have decreased transmembrane protein function and impaired signal transduction through G-protein coupled mechanisms. (245-246)

LR microdomains are lipid protein complexes in plasma membranes that are important in membrane transport and cellular signaling. These microdomains have a particular high concentration of PlsE, particularly with arachidonic acid in the sn2 position. Voltage gated potassium channel and Ca²⁺ channels are present in LR and can have their function impaired with changes in the LR lipid composition. (246)

4.4. Biological function

Many biological functions are attributed to Plasmalogens. The PlsE are known anti-oxidants, as they reduce the oxidation caused by ROS, metal ions and UV light. They exert can exert their anti-oxidant activity through their vinyl bond or by chelating metal ions. The vinyl ether bond prevents the oxidation of PUFA, which would lead to the formation of products that are less active. In fact, diseases characterized by diminished peroxissomal activity are known to be present cells more sensible to UV damage. On the other hand, hypochlorous acid, generated though the myeloperoxidase activity of activated leukocytes, attack plasmalogens generating α -chloro fatty aldehydes that are thought to increase leukocyte recruitment in inflammation and might also have a role in atherosclerosis. (245-246)

Moreover, plasmalogens are a reservoir of PUFA, due to the fact that FA in the sn2 position are usually of this class. By the action of a plasmalogens specific PLA2 PUFA can be released and exert their effect through eicosanoid and docosanoids synthesis. Also, ω 3 PUFA might also link to a G-protein coupled receptor, presenting an anti-inflammatory effect or even regulating adipogenesis. (245-246)

Plasmalogens have been linked with many diseases, especially diseases involving neurodegeneration and oxidative mechanisms. The levels of plasmalogens are decreased in diseases associated to peroxissomal disorders and altered levels/activity of peroxissomal enzymes as Zellweger's syndrome, cerebral adrenoleukodystrophy. In Alzheimer's disease, reduction in PlsE has been associated with the disease severity; besides that there is also present a 70 % reduction of PlsC in the frontal cortex. (246)

The plasmalogens levels, mainly PlsE, are decreased in plasma and in RBC from patients with metabolic diseases (as diabetes and obesity), cancer, hypertension, chronic kidney disease, atherosclerosis, and bronchopulmonary dysplasia. In obesity a diminished level of total plasmalogens, and of every plasmalogens species was reported. Plasmalogens serum levels are also reduced with IR. PlsC are reduced in open angle glaucoma, several years before the disease development. (249)

Both PlsE and PlsC are up regulated in membrane of myocardial cells of the diabetic heart, although there are changes in the relative plasmalogens species, leading to altered contractile function. (246)

4.5. Analytic procedures for plasmalogens

The substituent present in the sn1 position generates dimethylacetal (DMA) derivatives, when the FA analysis passes by derivation with the formation of FA methyl-esters (FAME). In this cases, the chromatogram will present, mainly, the palmitate dimethylacetal and octadecanal dimethylacetal, and, in a smaller proportion, octadecenal dimethylacetal, as plasmalogens vinyl ether derivatization product. (245-246)

5. Objectives

The objective of this study was to analyze the impact of obesity in the lipid profile, glucose homeostasis, inflammation and erythrocyte membrane composition, as well as, the cross-talk between the blood changes and the anthropometric data.

Our study focus with particular interest in the erythrocyte, as it is a cell with a limited defence and repair mechanisms, that accumulate the damage inflicted during its lifespan and, thus, could function as a marker for obesity related oxidative stress and inflammation.

6. Materials and methods

6.1. Subjects

The protocol used for all participants was approved by the Ethic Committees from the University Hospital S. João and from Children's Hospital Maria Pia, in Porto. Obese children and adolescents, aged 6-16 years, were identified from medical records, at the Pediatric Departments of the selected hospitals.

Obesity was defined as a BMI higher than the 95th percentile, for age and gender, according to the "2000 Centre for Disease Control and Prevention (CDC) growth charts". The BMIs of the control subjects were lower than the 85th percentile, adjusted for gender and age. Overweight was considered for BMI percentiles equal or higher than 85 and lower than 95.

Thirty four children participated in the study. This group was divided, according to the BMI percentile, in 17 obese, 8 overweight and 9 controls. Overweight and controls were age and sex matched with obese patients. All individuals were evaluated at starting of the study and comparisons between groups were performed (cross-sectional study).

All the children participated in the study after informed and written consent of their parents. Smokers, subjects under regular medication or with diabetes *mellitus*, endocrinologic disorders, hereditary diseases, inflammatory or infectious diseases were excluded from the study.

6.2. Procedures and assays

6.2.1. Clinical evaluation

Obtained the consent to participate in the study, children were invited to the research centers after an overnight fast, to collect blood and clinical data. The development of puberty was clinically assessed on the basis of Tanner stages. The physical evaluation included the measurement of height, weight, assessment of Tanner stage and observation to search for skin lesions related with obesity and its comorbidity.

Considering that BMI is not adjusted for sex and age, we used the body-mass index z-score (BMI z-sc) and the associated percentile of BMI to better define and identify the obese, overweight and controls individuals.

6.2.2. Blood samples

Blood samples were obtained on a fasting basis and processed within 2h of collection. Blood was obtained by venipuncture, to EDTA containing tubes.

Whole blood was used for hematologic studies, and, afterwards, used to obtain buffy-coat, plasma and RBC. Buffy coat and plasma was aliquoted and immediately stored at - 80°C until the assays. Rbc were used to prepare the RBC membranes for the proposed studies.

6.2.2.1. Basic hematological study

RBC count, Hb concentration, hematocrit (Ht) and hematimetric indexes, were evaluated by using an automatic blood cell counter (ABX Micros 60-OT, Horiva-ABX, France). Erythrocyte morphology was studied in Wright stained blood films.

6.2.3. Biochemical assays

6.2.3.1. Plasma lipids

Plasma lipids, lipoproteins and apolipoproteins analysis were performed in an auto-analyser (Cobas Mira S, Roche) using commercially available kits. Total cholesterol (Chol) and TG concentrations were determined by enzymatic colorimetric tests (CHOD-PAP and GPO-PAP methods, Roche, respectively). HDL-cholesterol and LDL-cholesterol levels were measured using enzymatic colorimetric tests after selective separation of HDL and LDL fractions (Direct HDLCholesterol and Direct LDLCholesterol, Roche). Apolipoprotein (apo) A-I and apo B levels were evaluated by immunoturbidimetric assays (uni-kit apolipoprotein A-I and B specific antisera, Roche).

Lipoprotein (a) (Lp(a)) plasma concentration was determined by an immunoturbidimetric method (Roche).

6.2.3.2. Glucose metabolism

The determination of circulating levels of glucose and insulin was performed by using routine automated technology (ABX Diagnostics).

Homeostasis model assessment insulin resistance (HOMA_{IR}) was used to detect the degree of insulin resistance (250), by using the following formula: resistance (HOMA_{IR}) = (fasting insulin ($\mu\text{U/ml}$) $\times$ fasting glucose (mmol/L)/22.5).

6.2.3.3. C-Reactive protein

Plasma levels of CRP were evaluated by immunoturbidimetry (CRP (latex) High-Sensitivity, Roche Diagnostics).

6.2.3.4. Bilirubin

Total plasma bilirubin concentration was evaluated using a commercially available kit (BIL-T; Roche, Basel, Switzerland).

6.2.4. Erythrocyte membrane studies

6.2.4.1. Preparation of membranes

RBC were separated from whole blood and washed twice in 0.9 % saline. Afterwards, RBC were submitted to hypotonic lysis, according to Dodge et al (251). The obtained membrane suspensions were carefully washed in Dodge buffer for four times, using in the first two washes a protease inhibitor, phenylmethylsulfonylfluoride (final concentration 0.1 mM in Dodge buffer). The protein concentration of the membrane suspensions was determined by Bradford's method (252).

6.2.4.2. Membrane bound hemoglobin

Membrane bound hemoglobin (MBH) was measured by spectrophotometry after protein dissociation of membrane components with Triton X-100 (5% in Dodge buffer) at 415 nm; the absorbance at this wavelength was corrected by subtracting the absorbance of the background at 700 nm; the absorbance value and the membrane protein concentration were then used to calculate the percentage of MBH.

6.2.4.3. Band 3 profile – electrophoretic separation of erythrocyte membrane proteins, immunoblot for Band 3 and densitometric evaluation

RBC membranes were treated with an equal volume of a solubilisation buffer containing 0.125 M Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoetanol, heat denatured and subjected to polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE) (20 µg of protein/lane) using the discontinuous Laemmli system (253). The slab gels were filled with a 4.5% polyacrylamide stacking gel and a 9.0% polyacrylamide separating gel. Proteins were electrophoretically transferred from the SDS-PAGE gels to a nitrocellulose sheet (0.2 µm porosity, Sigma). Additional reactive sites on the nitrocellulose were blocked by incubation in a low fat milk phosphate buffered solution (PBS), pH 7 (5% in 0.1% Triton X-100), overnight at 4°C, under gentle rotation. The Band 3 immunoblot was then performed to determine whether erythrocyte Band 3 was modified; monoclonal antibodies anti-human Band 3, produced in mouse, recognizing an epitope located in the cytoplasmic domain of the Band 3 molecule (Sigma) were added (1:3000) and incubated for 4 h; washing of the nitrocellulose was followed by the addition and incubation with anti-mouse Ig peroxidase linked (Sigma) for 2 h (1:4000).

The incubations were carried out at room temperature; the dilutions of the antibodies were prepared with PBS pH 7.0, containing 0.1% Triton-X 100 and 0.5% low fat dry milk. The washes used the same buffer, without low fat dry milk. The development of the immunoblot was performed by adding hydrogen peroxide and α-chloronaphtol (Sigma) (81). The immunoblots were scanned (Darkroom CN UV/wl, BioCaptMW version 99, Vilber Lourmat, Marne-La –Valle´ e, France) and the relative amount of Band 3 monomer (Mon), high molecular aggregates (HMWAg) and of proteolytic fragments (Frag), were quantified by densitometry (Bio1Dqq version 99, Vilber Lourmat, Marne-La –Valle´ e, France).

6.2.4.4. Membrane lipoperoxidation

RBC membrane lipid peroxidation was determined using the thiobarbituric acid (TBA) test according to Mihara and Uchiyama with some modifications. (254)

The membrane suspension was homogenized and 100 µl were added to 360 µl of 1% H₃PO₄ (v/v) and 120 µl of 0.6% TBA (w/v), and heated in boiling water for 45 minutes. After immersion in ice for 10 minutes, MDA was extracted with butanol (480 µl) and after centrifugation, the absorbance of the supernatant was measured by spectrophotometry (535 and 520 nm). The ratios of MDA/protein concentration (mg) for each RBC membrane suspension sample were calculated to avoid bias of the results (due to the protein concentration of the membrane suspension used).

6.2.4.5. Membrane protein carbonylation

The method used to measure protein carbonylation (PC) was adapted from Levine *et al.* (255) Briefly, 50 µl of membrane suspensions were mixed with 200 µl of 2.5M HCl (blank) or with 200 µl of 10mM 2,4-dinitrophenylhydrazine DNPH (test), and incubated in the dark, at room temperature, for 1 hour, mixing every 15 minutes. Afterwards, membrane pellets were resuspended with 250 µl of 20% trichloroacetic acid (TCA) (w/v) and immersed in ice for 10 minutes. This step was repeated with 10% TCA (w/v). After washing (3 times) with ethanol:ethyl acetate mixture 1:1, the protein pellets were re-suspended in 500 µl of the washing mixture and incubated at 37°C for 10 minutes. PC was measured by spectrophotometry (370 nm) and the molar extinction coefficient ($\epsilon=22000\text{M}^{-1}\text{cm}^{-1}$) was used to calculate its concentration. Results were expressed as PC (PM) per protein concentration of the membrane suspension (mg).

6.2.4.6. Analysis of membrane fatty acids

6.2.4.6.1. Lipid extraction

The membrane's lipids were extracted using and adaptation of the Folch method. (256) Briefly, 200 µl of the membrane suspension were centrifuged to remove the suspending buffer, and afterwards 50 µl of 0.1% BHT (butylhidroxytoluene), to prevent lipid oxidation during processing, and 950 µl of methanol were added; samples were vortexed and

subjected to ultrasounds for 10 minutes. Then, 2 ml of chloroform were added, the sample was homogenised by vortex mixing and left over-night at 4°C in the dark. Subsequently, 500 µl of KCl solution (0.88%) were then added and the sample homogenised and centrifuged. The upper phase was eliminated by vacuum aspiration and the remaining water residues eliminated with anhydrous sodium sulphate. After centrifugation, the liquid extract was removed and dried under nitrogen stream (40°C).

6.2.4.6.2. Derivatization

The process used, triggered the formation of FAME. (27) The derivatization reagent (800 µl of a 1:23 acetyl chloride:methanol) was added to the dried samples, thoroughly mixed and heated at 80°C in an oven for 2 hours. After cooling down, 900 µl of n-hexane was added to the sample that was mixed and again centrifuged. The upper phase was recovered and the extraction process repeated with an equivalent portion of n-hexane. The extracts were combined in a second vial and dried at 40°C under a nitrogen stream. The dried sample was recovered with 60 µl of dichloromethane and transferred to a glass insert (200 µl). The samples were stored at -20°C until injection (no more than two days).

6.2.4.6.3. Chromatographic analysis

The chromatographic analysis were performed in a gas chromatography with flame ionization detector (GC-FID) chromatograph from Chrompack (CP-9001), equipped with an automatic injection sampler (CP-9050). The following conditions were used:

- Column: CP-Sil 88 of 50 m x 0.25mm (0.19 µm)
- Oven: 140°C – 5 min, 5 °C / min until 200 °C (15min)
- Injector: 250 °C
- Detector: Flame ionization detector - 270 °C
- Gas: He – 110 kPa

The injections were performed in a split-less mode. Each sample was at least injected twice.

A commercial standard mix (Supelco-37 FAME Mix) was used for peak comparison and FID calibration.

6.2.4.6.4. Chromatogram analysis

The FA were identified in the chromatogram by comparison with the Supelco FAME standard. FA FAME that were not present in the Supelco mix were identified according to their relative chromatographic position/amounts and available literature. All FA areas were corrected for the FID calibration responses obtained with the Supelco-37 mix. Their individual relative percentages were calculated on the basis of the total chromatographic area between C14:0 and C22:6n3.

Table 1 lists the FA that were identified and used for statistic analysis. Although other peaks were present in some samples, their irregularity and / or fluctuations in the values did not allow a correct identification.

Table 1. List of identified fatty acids and Supelco 37 standard related FAME.

Fatty Acid		Supelco 37
Myristic	14:0	X
Pentadecanoic	15:0	X
Hexadecanal dimethylacetal	16:0 DMA	
Palmitic	16:0	X
7-Hexadecenoic	16:1n9	
Palmitoleic	16:1n7	X
Margaric	17:0	X
Octadecanal dimethylacetal	18:0 DMA	
Octadecenal dimethylacetal	18:1 DMA	
Stearic	18:0	X
Elaidic ¹	18:1t	X
Oleic	18:1n9	X
cis-Vaccenic	18:1n7	
Linoelaidic ²	18:2t	X
Linoleic	18:2n6	X
Eicosanoic	20:0	X
α -Linolenic	18:3n3	X
Eicosadienoic	20:2n6	X
Behenic	22:0	X
Dihomo- γ -linolenic	20:3n6	X
Arachidonic (AA)	20:4n6	X
Tricosylic	23:0	X
Docosadienoic	22:2n6	X
Eicosapentaenoic (EPA) ³	20:5n3	X
Lignoceric ³	24:0	X
Nervonic	24:1n9	X
Docosatetraenoic	22:4n6	
Docosapentaenoic (DPAn6)	22:5n6	
Tetracosodienoic	24:2n6	
Docosapentaenoic (DPAn3)	22:5n3	
Docosahexaenoic (DHA)	22:6n3	X

FAME, fatty acid methyl ester. ¹All oleic acid trans isomers were added together for statistical analysis. ²All linoleic acid trans isomers were added together for statistical analysis. ³The chromatographic column did not separate the FAs eicosapentaenoic and lignoceric. For statistical analysis these two fatty acids were considered together, lignoceric acid being the major FA. All results were analysed taking this fact into account.

The individual FAs were grouped according to similar structural characteristics. They were also used to calculate ratios that provide information about FAs relative balance that can be used to estimate enzyme activity. Table 2 lists the groups and ratios used in this work to better understand the FAs dynamics and relation with other variables studied.

Table 2. Groups of fatty acids and ratios.

Groups and Ratios	Definition
SFAs (%)	Sum of individual SFA %
UFAs (%)	Sum of individual UFA %
UFAs/SFAs (%)	
MUFAs (%)	Sum of individual MUFA %
LCPUFAs (%)	Sum of individual PUFA % with carbon chain ≥ 22 carbon
n3PUFAs (%)	Sum of individual n3 PUFA %
n6PUFAs (%)	Sum of individual n6 PUFA %
(n3/n6)PUFAs	
DHAn3/DPAn6	
Trans (%)	Sum of individual trans FA %
DMA (%)	Sum of individual DMA %
15:0+17:0 (%)	
n3 Product / precursor	$(22:5+22:6)/n3/18:3n3$
$\Delta 5$	$20:4n6/20:3n6$
$\Delta 6$	$20:3n6/18:2n6$
$\Delta 9^a$	$18:1n9/18:0$
$\Delta 9^b$	$16:1n7/16:0$
Elongase ^a	$18:0/16:0$
Elongase ^b	$22:4n6/20:4n6$
n3 series	$22:6n3/18:3n3$
n6 series	$20:4n6/18:2n6$
UI	Σ (individual fatty acids % * number of unsaturations per fatty acids)
EFA def	$16:1n7/18:2n6$

FA, Fatty acid; n3, n 3 FA family; n6, n6 FA family; SFA, saturated FA; UFA, unsaturated FA; PUFA, polyunsaturated FA; LCPUFA, long chain PUFA; Trans, trans FA, DMA, dimethyl acetyl FA derivative; EFA, Essential FA; EFA def, EFA deficiency ratio; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; Δ , delta desaturase; UI, unsaturation index.

6.3. Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS, version 19.0), for Windows. Kolmogorov-Smirnov analysis was used to test if the results were normally distributed. The results normally distributed are presented as mean $\pm$ standard deviation and those not-normally distributed are presented as median (interquartile range).

Whenever parameters did not present a Gaussian distribution, the variables were subject to transformation, prior to comparison between groups. To compare the three groups Oneway Anova Tukey *post hoc* test was used.

Pearson's correlation coefficient was used to evaluate relationships between sets of data.

Linear regression analysis was performed to identify the best explanatory models.

A P-value < 0.05 was considered as statistically significant.

7. Results and discussion

The studied population was divided in three groups: Control ($n = 9$), Overweight ($n = 8$) and Obese ($n = 17$), according to the BMI percentile, adjusted to age and sex (2002 CDC Growth Charts).

- Control: $15 < \text{BMI percentile} < 85$.
- Overweight: $85 \leq \text{BMI percentile} < 95$.
- Obese: $\text{BMI percentile} \geq 95$.

No differences were found between groups for age, sex, height and Tanner distribution. The adjustment of groups for gender and age is important, as some biochemical variables might be influenced by these two factors. The clinical characterization of the population is presented in table 3.

Table 3. Clinical and antropometric characteristics of the studied groups.

	Control ($n=9$)	Overweight ($n=8$)	Obese ($n=17$)
Age (years)	15.2 $\pm$ 1.2	14.4 $\pm$ 1.3	13.4 $\pm$ 2.8
Sex (Male(%))	3 (33.3)	3 (26.7)	9 (52.9)
Weight (kg)	59.4 $\pm$ 8.5	61.7 $\pm$ 7.5	82.2 $\pm$ 20.7 §
Height (cm)	164.0 $\pm$ 6.8	157.1 $\pm$ 5.8	157.8 $\pm$ 11.9
Waist (cm)	78.5 $\pm$ 7.0	86.6 $\pm$ 4.4 ¥	97.8 $\pm$ 10.6 §
Waist/Height	0.488 $\pm$ 0.040	0.552 $\pm$ 0.032 ¥	0.620 $\pm$ 0.057 §
BMI (kg/m²)	21.98 $\pm$ 1.95	24.95 $\pm$ 2.01	32.49 $\pm$ 5.37 §
BMI z-score	0.54 $\pm$ 0.44	1.32 $\pm$ 0.18 ¥	2.25 $\pm$ 0.28 §
Percentile	69.2 $\pm$ 15.5	90.4 $\pm$ 3.4 ¥	98.5 $\pm$ 1.0 §

¥: $P < 0.05$ vs. Control; §: $P < 0.05$ vs. both groups. BMI, body mass index.

We observe a significant increase in weight, waist, waist/height ratio (W/H), BMI and BMI z-sc from the control to the obese group.

The results clearly show that overweight is already associated with significant features of adiposity that are even more enhanced in the obese group.

7.1. Lipid profile

The study of blood lipids showed that the obese group presented the worst profile, as compared to the other two groups. The overweight group did not show significant differences, as compared to the control group. The obese group presented a significant increase in TG, Chol (vs. overweight), LDL (vs. control), apo B and Chol / HDL (vs. both groups). A trend to higher levels of Lp (a) and lower values of HDL was present in obese individuals (Table 4).

Table 4. Lipid profile of the studied groups.

	Control (n=9)	Overweight (n=8)	Obese (n=17)
TG (mg/dl)	54.0 (49.5-62.5)	45.0 (35.2-57.5)	68.0 (48.0-112.5) £
Chol (mg/dl)	134.0 (130.5-169.0)	134.5 (109.5-168.0)	172.0 (135.5-187.5) £
HDL (mg/dl)	68.0 (63.0-70.0)	65.5 (62.2-67.8)	63.0 (55.5-67.0)
LDL (mg/dl)	93.0 (78.5-135.0)	102.5 (73.0-151.2)	165.0 (114.5-182.0) ¥
Lp (a) (mg/dl)	19.5 (14.5-80.6)	26.2 (8.3-35.6)	42.1 (25.7-71.4)
Apo A (mg/dl)	116.3 ± 9.8	102.1 ± 12.2	103.1 ± 17.5
Apo B (mg/dl)	46.2 ± 15.6	42.8 ± 17.0	62.3 ± 11.5 §
Chol/HDL	2.22 (1.95-2.57)	2.10 (1.75-2.56)	2.83 (2.32-2.94) §
Apo A/Apo B	3.18 ± 2.49	3.09 ± 2.36	1.72 ± 0.45

¥: $P < 0.05$ vs. Control; £: $P < 0.05$ vs. Overweight; §: $P < 0.05$ vs. both groups. TG, triglycerides; Chol, total Cholesterol; HDL, high density lipoprotein Cholesterol; LDL, low density lipoprotein Cholesterol; Lp, lipoprotein; apo, apolipoprotein.

It is important to highlight that clear risk changes in the lipid profile are already present in such early ages. A risk lipid profile has been associated with obesity, which is a well known risk factor for CVD. (3-4) In fact, the increase of LDL and the decrease of HDL are related to atherogenesis and increased carotid arteria thickness. (257-258) Besides the absolute number of LDL particles in circulation their relative size should also be taken into account. Obesity is associated with smaller LDL particles that are considered to be more oxidable and atherogenic than larger particles. (258)

Raised TG are also considered as a CV risk factor, by modulating the formation of small LDL. (259)

Atherosclerosis is known to begin early in life. Atherosclerotic changes have already been identified in the arteries of adolescents. (6) Thus, it is crucial to act early in an individual's life and prevent the establishment of obesity and its metabolic consequences.

7.2. Glucose metabolism and Inflammation

To evaluate some of the metabolic changes, we analyzed glucose metabolism and CRP as an important, sensitive marker of inflammation. (259-260)

The group of overweight did not present changes in glucose metabolism, as compared to control. However, the obese group presented significant changes in glucose metabolism, when compared to control, namely significantly higher levels of circulating insulin and a significantly higher HOMA_{IR}, a marker of IR; no difference was found for glucose plasmatic concentration (Table 5).

Table 5. Glucose metabolism and C-reactive protein in the studied groups.

	Control (n=9)	Overweight (n=8)	Obese (n=17)
Glycemia (mg/dl)	92.0 (83.5-96.0)	90.5 (85.2-99.5)	90.0 (84.5-93.5)
Insulin (μU/ml)	7.48 (6.06-9.94)	9.93 (7.11-11.08)	12.98 (9.50-20.34) ¥
HOMA_{IR} ((μU*mmol)/l²)	1.69 (1.34-2.34)	2.10 (1.59-2.49)	2.95 (2.02-4.30) ¥
CRP (mg/l)	0.27 (0.20-0.78)	0.73 (0.41-1.36)	2.47 (1.14-9.89) §

¥: $P < 0.05$ vs. Control; §: $P < 0.05$ vs. both groups. HOMA_{IR}, homeostasis model assessment - insulin resistance; CRP, C-reactive protein.

The increased insulin level without significant change in glycemia, suggests a disturbance of glucose homeostasis. The reason for this unbalance may be due to an impaired glucose stimulated insulin secretion, but, it seems to be mainly the result of a peripheral resistance to insulin action, a traditional feature in obesity. This peripheral IR would lead to the need for increasing insulin secretion, to produce the same glucose lowering effect. (261-262) The impaired balance between glucose and insulin is clarified by the increased HOMA_{IR}. Childhood obesity seems to increase the probability of developing T2DM in adulthood, which is usually proceeded by metabolic changes, as a reduced insulin sensitivity. (263)

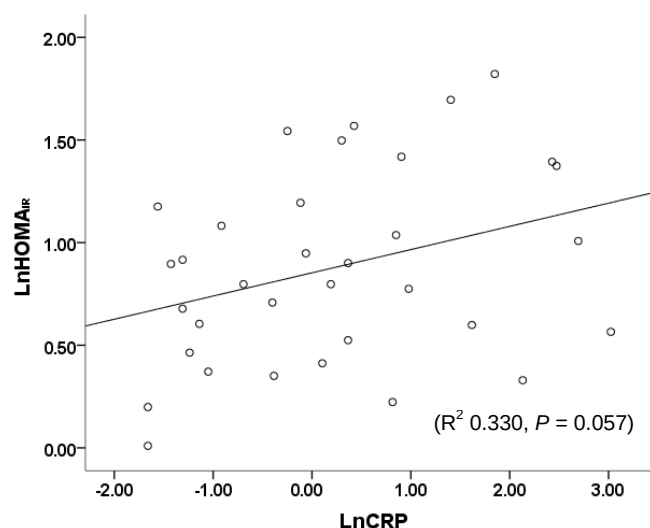
Obesity is considered a state of low-grade chronic inflammation. (10-12) In fact, the growing adiposity is linked to increased levels of circulating pro-inflammatory adipokines and a reduction in anti-inflammatory mediators, as adiponectin. (6-8)

In our studied population, CRP, a traditional and sensitive marker of inflammation, was significantly increased in obese when compared to both control and overweight (almost 8 fold the control value) showing the low grade inflammatory process. (Table 5)

Different studies used different CRP cut-off levels to establish an increased risk for CVDs. Some studies defined a value of 3 mg/l, while others considered a lower value of 1 mg/l. (259-260) In our study only the obese individuals presented a median CRP value higher than 1 mg/l. However, as already referred, there are significant differences between the 3 groups (control, overweight and obese), reflecting an inflammatory process.

There is an association between inflammation, IR and dyslipidemia. These three variables, as well as obesity, underlie the development of many diseases, especially CVDs. (3-4) The increase in HOMA_{IR} and CRP with increasing BMI percentile is shown in figure 1. Although there is a clear trend to an increase in IR with increasing CRP values, the correlation between CRP and HOMA_{IR} reached only borderline statistical significance (R^2 0.330, P = 0.057), probably due to the small sample size.

Figure 43. LnCRP and LnHOMA_{IR} correlation.



CRP, C-reactive protein; HOMA_{IR} , homeostasis model assessment - insulin resistance

7.3. Erythrogram and markers of erythrocyte damage

With increasing adiposity there is an increase in the blood demand by the adipose tissue. (11) In fact, obesity is associated with an increase in angiogenic mediators, e.g. vascular endothelial growth factor (VEGF), and with an erythropoietic stimulus. (12, 264) As the adipose tissue grows there are probably cells that become more distant from the circulation and, consequently, an hypoxic environment develops. This hypoxic milieu activates the hypoxia inducible factor 1 (HIF-1) that would lead to an increase in local and circulating angiogenic factors and to accelerated erythropoiesis. (265) This angiogenic stimulus and enhanced erythropoiesis are linked to an increase in oxidative stress. (264, 266) Ultimately, the oxidative stress can cause damage in membrane lipids and proteins from different tissues and cells, as the RBC that is a particular cell, with poor repair mechanisms and biosynthesis capacity. (19-20) Due to this characteristics, we used the RBC as a good model to estimate cumulative damage in the cell.

The three groups presented no differences regarding the erythrogram and markers of erythrocyte membrane damage (Table 6). Indeed, no difference in oxidative damage markers was found for overweight and obese, when compared to control. It has been reported that in some oxidative states, such as pregnancy, presenting simultaneously oxidative stress and an erythropoietic stimuli, the coexistence of older damaged cells with younger erythrocytes, may obscure the oxidative damages. (21) Our data suggest that at this age the influence of obesity in RBC profile is not yet evident. The study of reticulocytes could offer important information about a possible erythropoietic compensation of RBC senescence.

Table 6. Erythrogram and markers of membrane damage.

	Control (n=9)	Overweight (n=8)	Obese (n=17)
RBC ($\times 10^9/l$)	4.82 $\pm$ 0.28	4.89 $\pm$ 0.36	4.89 $\pm$ 0.36
Hb (g/dl)	13.9 $\pm$ 1.2	13.6 $\pm$ 0.7	14.0 $\pm$ 1.0
Ht (l/l)	0.412 $\pm$ 0.036	0.406 $\pm$ 0.016	0.407 $\pm$ 0.029
MCV (fl)	84.7 $\pm$ 5.6	86.0 $\pm$ 2.5	83.3 $\pm$ 4.0
MCH (pg)	28.8 $\pm$ 1.6	28.8 $\pm$ 0.9	28.6 $\pm$ 1.7
MCHC (g/dl)	33.8 $\pm$ 0.5	33.4 $\pm$ 0.8	34.4 $\pm$ 1.0 £
RDW (%)	14.79 $\pm$ 0.87	14.84 $\pm$ 0.63	14.91 $\pm$ 0.67
Bilirrubine (mg)	0.38 (0.26-0.50)	0.38 (0.28-0.44)	0.27 (0.22-0.36)
Ag (%)	17.40 $\pm$ 10.53	20.61 $\pm$ 8.38	18.92 $\pm$ 6.65
Mon (%)	57.45 $\pm$ 22.44	66.44 $\pm$ 8.20	69.63 $\pm$ 5.06
Frag (%)	14.04 $\pm$ 9.53	12.95 $\pm$ 9.95	10.86 $\pm$ 6.50
MBH (% $\times 10^{-4}$)	39.0 (35.0-44.5)	43.0 (39.8-50.8)	39.0 (35.0-41.5)
MDA/prot (mg)	0.0192 $\pm$ 0.0092	0.0156 $\pm$ 0.0041	0.0172 $\pm$ 0.0099
Prot Carb/prot (mM/mg)	2.54 $\pm$ 2.16	1.40 $\pm$ 0.71	2.29 $\pm$ 0.92

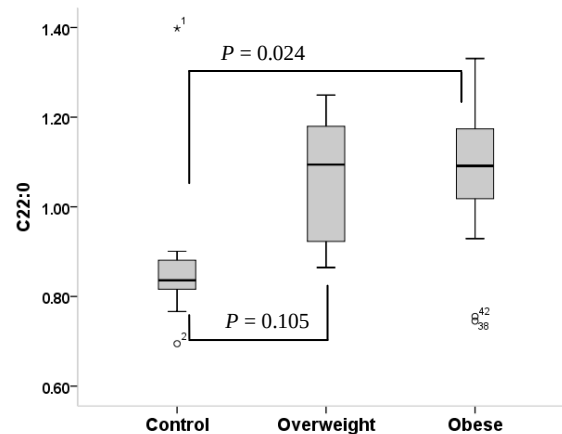
£: $P < 0.05$ vs. Overweight. RBC, red blood cell; Hb, hemoglobin; Ht, hematocrit; MCV, mean cellular volume; MCH, mean cellular hemoglobin; MCHC, mean cellular hemoglobin concentration; RDW, red blood cells distribution width; Ag, high molecular weight aggregates; Mon, monomers; MBH; membrane bound hemoglobin; MDA, malonaldehyde; PC, protein carbonylation.

7.4. Membrane fatty acids

The membrane FA profiles of the participants in this study are in agreement with other studies made on erythrocytes. (237, 267-268)

Obesity is often associated to unbalanced diets and to a sedentary behavior. (13, 137) Diets from obese individuals are usually rich in SFA and poor in PUFA, especially in n3 PUFA. (142) It is also frequent an increased consumption of trans FA. (145-146) This type of dietary habits, together with endogenous changes in FA and lipid metabolism, lead to changes in the proportions of the different FA in body tissues. (139, 267)

The profile of FA in the erythrocyte membrane in obese individuals usually presents an increase of SFA, and a reduction in PUFA, especially in n3 PUFA. In this way, there is a reduction of the membrane unsaturation level. (83-84) In agreement, the membrane fatty acid profile of our obese and overweight individuals presented a significantly increase in 22:0 (vs. controls). (Figure 44)

Figure 44. Behenic acid in the erythrocyte membranes for the studied groups.

Also a trend towards increasing levels of saturated odd numbered FA 15:0 and 17:0 was found (Table 7). Animal fat, particularly from dairy products, are a good source of these FA. (131, 152) Therefore, an increased consumption of these products, rich in saturated fats, may contribute to the observed increase in FA 15:0 and 17:0, and favor the development of obesity.

Overweight individuals presented significantly higher levels 22:4n6 in the RBC membrane, when compared to controls. An increasing proportion of 20:0, 18:3n3 and 20:3n6 were found for overweight and obese individuals, although without statistical significance. The 22:4n6 FA was significantly higher in overweight, as compared to controls, and still higher in obese, though without statistical significance. The increase in n6 PUFA is in agreement with some authors that point to a rebound mechanism of the body to maintain the membrane unsaturation and its rheological properties by increasing the proportion of n6 PUFA. (84) The reason for this increase in n6 FA might be due to their increased availability in modern diets in comparison to n3 PUFA. (13, 137)

The complete studied fatty acid profile for each group is presented in table 7.

Table 7. Fatty acid profile observed in the erythrocyte membranes of the studied groups.

Fatty acid	Control (n=9)	Overweight (n=8)	Obese (n=17)
14:0 (%)	0.364 ± 0.081	0.410 ± 0.192	0.380 ± 0.169
15:0 (%)	0.203 ± 0.084	0.238 ± 0.209	0.376 ± 0.226
16:0 DMA (%)	2.114 ± 0.315	2.093 ± 0.258	2.073 ± 0.299
16:0 (%)	21.194 ± 1.529	19.859 ± 1.280	20.094 ± 1.182
16:1n9 (%)	0.116 ± 0.044	0.151 ± 0.047	0.133 ± 0.050
16:1n7 (%)	0.267 ± 0.082	0.263 ± 0.101	0.279 ± 0.092
17:0 (%)	0.286 ± 0.046	0.301 ± 0.044	0.307 ± 0.037
18:0 DMA (%)	2.729 ± 0.438	2.844 ± 0.284	2.937 ± 0.501
18:1 DMA (%)	0.579 ± 0.119	0.626 ± 0.081	0.574 ± 0.103
18:0 (%)	18.275 ± 1.849	17.219 ± 1.313	17.267 ± 1.138
18:1t (%)*	0.250 ± 0.148	0.212 ± 0.104	0.189 ± 0.088
18:1n9 (%)	9.984 ± 1.364	10.318 ± 1.694	10.300 ± 1.468
18:1n7 (%)	0.692 ± 0.118	0.680 ± 0.083	0.698 ± 0.136
18:2t (%)*	0.353 ± 0.155	0.295 ± 0.228	0.408 ± 0.199
18:2n6 (%)	9.082 ± 0.400	8.598 ± 0.702	8.606 ± 1.111
20:0 (%)	0.250 ± 0.047	0.272 ± 0.036	0.283 ± 0.057
18:3n3 (%)	0.158 ± 0.023	0.170 ± 0.017	0.176 ± 0.044
20:2n6 (%)	0.302 ± 0.052	0.279 ± 0.033	0.316 ± 0.061
22:0 (%)	0.886 ± 0.201	1.063 ± 0.150	1.085 ± 0.167 ¥
20:3n6 (%)	1.763 ± 0.542	1.887 ± 0.421	2.098 ± 0.488
20:4n6 (%)	12.254 ± 1.306	12.728 ± 0.838	12.315 ± 0.973
23:0 (%)	0.377 ± 0.075	0.412 ± 0.168	0.322 ± 0.182
22:2n6 (%)	0.094 ± 0.052	0.081 ± 0.012	0.140 ± 0.074
20:5n3 + 24:0 (%)	2.810 ± 0.555	3.049 ± 0.409	3.100 ± 0.334
22:4n6 (%)	2.002 ± 0.228	2.427 ± 0.320 ¥	2.272 ± 0.345
24:1n9 (%)	3.253 ± 0.700	3.309 ± 0.395	3.348 ± 0.575
22:5n6 (%)	0.647 ± 0.075	0.670 ± 0.082	0.674 ± 0.226
24:2n6 (%)	0.461 ± 0.116	0.441 ± 0.122	0.368 ± 0.182
22:5n3 (%)	1.432 ± 0.206	1.420 ± 0.136	1.520 ± 0.225
22:6n3 (%)	4.322 ± 0.701	4.575 ± 0.517	4.305 ± 0.556

¥: $P < 0.05$ vs. Control. *Sum of all identified trans isomer of oleic acid. Named is the most important trans isomer (Elaidic acid). ** Sum of all identified trans isomers of linoleic acid. Named is the most important trans isomer (Linoelaidic acid).

After the individual analysis of the FA from the RBC membrane, we have separated the FA in groups, according to their characteristics, such as the presence, number and type of unsaturations, the ω family and their relation to dietary habits. The ratios between different

FA were also calculated. These ratios are associated with the activity of important enzymes in FA metabolism, e.g. desaturases and elongases. The results from the different ratios should be considered carefully. Although they can give an estimation of an enzyme activity, there are other factors that can influence the FA proportions in the RBC membrane, being diet the first one. The dynamic relations between the several types of FA studied are presented in table 8.

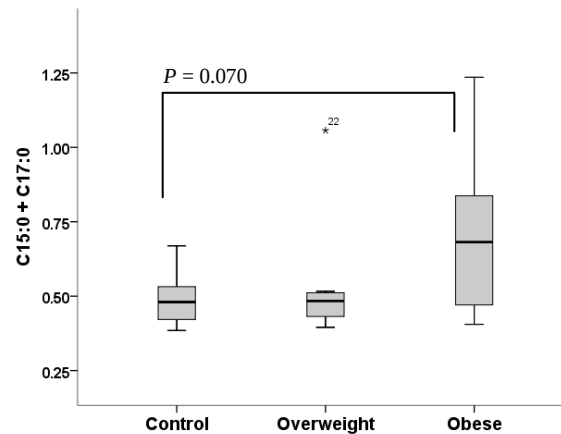
Table 8. Proportions of different type of fatty acids for the studied groups.

	Control (n=9)	Overweight (n=8)	Obese (n=17)
SFA (%)	41.847 ± 3.072	39.775 ± 1.562	40.115 ± 0.862
UFA (%)	46.959 ± 2.127	48.277 ± 1.368	47.788 ± 0.940
UFA/SFA (%)	1.130 ± 0.123	1.216 ± 0.081	1.192 ± 0.043
MUFA (%)	14.312 ± 1.492	14.720 ± 1.629	14.748 ± 1.398
LCPUFA (%)	32.741 ± 1.712	33.677 ± 0.949	33.325 ± 1.357
n3PUFA (%)	5.912 ± 0.766	6.166 ± 0.600	6.001 ± 0.542
n6PUFA (%)	26.604 ± 1.610	27.111 ± 0.770	26.788 ± 1.134
(n3/n6)PUFA	0.223 ± 0.033	0.228 ± 0.024	0.224 ± 0.022
DHAn3/DPAn6	6.785 ± 1.479	6.919 ± 1.103	7.285 ± 3.457
15:0 + 17:0 (%)	0.489 ± 0.092	0.538 ± 0.214	0.684 ± 0.240
n3 Prod/Prec	36.961 ± 6.187	35.524 ± 4.442	35.165 ± 9.778
Δ 5	7.394 ± 1.902	7.065 ± 1.698	6.190 ± 1.549
Δ 6	0.195 ± 0.062	0.221 ± 0.053	0.249 ± 0.070
Δ 9 ^a	0.553 ± 0.108	0.608 ± 0.154	0.602 ± 0.118
Δ 9 ^b	0.0126 ± 0.0035	0.0132 ± 0.0048	0.137 ± 0.0039
Elongase ^a	0.862 ± 0.058	0.871 ± 0.097	0.864 ± 0.098
Elongase ^b	0.164 ± 0.019	0.191 ± 0.024	0.185 ± 0.029
n3 series	27.752 ± 5.294	27.112 ± 3.679	26.067 ± 7.744
n6 series	1.355 ± 0.188	1.494 ± 0.209	1.460 ± 0.267
UI	133.3 ± 7.7	138.2 ± 4.5	135.6 ± 4.417
EFA def	0.0294 ± 0.0089	0.0304 ± 0.0102	0.0331 ± 0.0120
Trans (%)	0.603 ± 0.275	0.508 ± 0.302	0.598 ± 0.237
DMA (%)	5.432 ± 0.786	5.534 ± 0.570	5.584 ± 0.817

FA, Fatty acid; n3, n 3 FA family; n6, n6 FA family; SFA, saturated FA; UFA, unsaturated FA; EFA, Essential FA; EFA def, EFA deficiency ratio; PUFA, polyunsaturated FA; LCPUFA, long chain PUFA; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; Prod/Prec, Product/precursor; Δ, delta desaturase; UI, unsaturation index; Trans, trans FA, DMA, dimethyl acetyl FA derivative. n3 Product/precursor ratio, (22:5+22:6)n3/18:3n3; Δ5 desaturase, 20:4n6/20:3n6; Δ6 desaturase, 20:3n6/18:2n6 ratio; Δ9^a, 18:1n9/18:0; Δ9^b, 16:1n7/16:0; Elongase^a, 18:0/16:0; Elongase^b, 22:4n6/20:4n6; n3 Series, 22:6n3/18:3n3; n6 Series, 20:4n6/18:2n6 ratio; UI, Σ (individual fatty acids % * number of unsaturations per fatty acids); EFA def, 16:1n7/18:2n6.

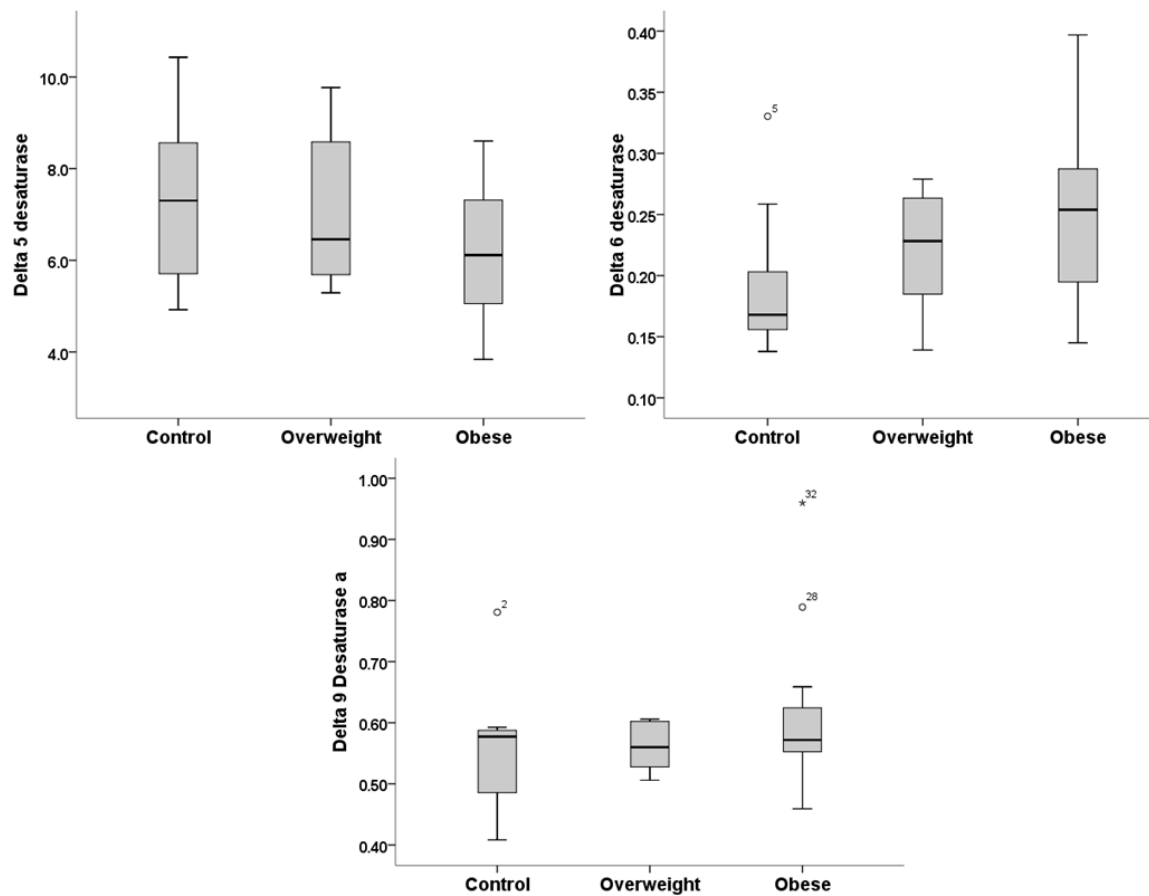
Both overweight and obese group presented a trend towards the reduction in the UFA and increase in SFA in RBC membranes, when compared to control. However, the obese group presented a trend to higher values of 15:0 plus 17:0 SFA, in harmony to the findings por individual FA. (Figure 45)

Figure 45. Odd numbered FA 15:0 and 17:0 vs. BMI percentile groups



Concerning the activities of the desaturases ($\Delta 5$, $\Delta 6$, $\Delta 9$), no significant differences were identified between groups. Nevertheless, there is a trend towards a lower activity of the $\Delta 5$ desaturase (20:4n6/20:3n6) and for a higher activity of $\Delta 6$ desaturase (20:3n6/18:2n6) and $\Delta 9b$ (16:1n7:16:0), with increasing BMI percentile. (Figure 46)

Figure 46. Ratios and enzymes activities of $\Delta 5$, $\Delta 6$ and $\Delta 9$ desaturases, for the studied groups.



7.5. Linear regression analysis

To better study the impact of each FA, of their relative proportions and of the derived ratios in the different variables we performed, a linear regression analysis. The part of a variable fluctuation that is explained by the proposed model is indicated by R^2 , being one (1) the maximum possible value.

In a first approach, the studied markers of adiposity were used as the dependent variables: BMI, BMI z-sc, waist-to-height (W/H) ratio and weight. The following models were defined:

7.5.1. Body mass index

Table 9. Linear regression models with markers of adiposity as the dependent variables and fatty acids as independent variables.

DepV	IndV	B	SE	SB	Pv	R ²	aR ²	Pm
BMI	Constant	-8.519	5.696		.146	.460	.425	<.001
	15:0	7.316	3.639	.243	.054			
	22:0	13.267	3.729	.404	.001			
	18:3n3	68.308	19.802	.380	.002			
	20:3n6	4.612	1.401	.370	.003			
BMI z-sc	Constant	-2.057	.717		.007	.460	.425	<.001
	22:0	2.237	.557	.530	.000			
	20:3n6	.683	.212	.425	.003			
W/H	Constant	.113	.076		.147	.561	.517	<.001
	22:0	.217	.049	.537	.000			
	20:3n6	.066	.019	.430	.001			
	18:3n3	.593	.272	.268	.037			
Weight	Constant	23.952	12.925		.073	.435	.399	<.001
	15:0	43.754	12.741	.475	.002			
	18:3n3	202.115	76.142	.367	.012			

DepV, dependent variable; IndV, independent variable; B, coefficient beta; SE, standard error; SB, stardartized B; Pv, levels of significance of the variable; R², squared correlation coefficient; aR², adjusted R²; Pm, level of significance of the model. BMI, body mass index; BMI z-scc, BMI z-score; W/H, waist-to-height ratio.

The W/H seems to be the adiposity variable which variation was the most explained by an individual FA model (56.1%); BMI and BMI z-sc had 46.0% of their variation explained by the FA model and weight only 43.5 %. (Table 9).

As the models show, the FA 15:0, 22:0, 18:3n3 and 20:3n6 are significant determinants of the obesity markers, which is in agreement with the differences and trends already noted for the FA between BMI percentiles groups.

It is notable the importance of docosanoic acid (22:0) in all obesity markers, excluding weight. SFA consumption have been linked with increased body weight by many studies, being their relative proportions increased in body tissues. (13, 137)

Both DGLA (20:3n6) and ALA (18:3n3) are also determinants of obesity. The positive relation between these PUFA and the markers of adiposity is interesting, as they were proposed to have anti-inflammatory properties, and in our obese group we found a

significant increase in CRP. Some authors reported an increase in n6 PUFA, as a body reaction to the decrease in membrane unsaturation, especially due to the reduction in n3 PUFA. (84) However, in our population this does not appear to be the case, as 18:3n3 is increased and there is no change in 22:5n3 and 22:6n3.

BMI is the adiposity measure that includes the more determinants, a total of four FA. It is quite interesting to see that the model constructed for BMI appears to be somehow divided between the models for BMI z-sc and weight. In this way, the FA docosanoic and DGLA appear to be more related to BMI z-sc, BMI adjusted for sex and age, while ALA and 15:0 appear to be closely related to the absolute body mass (weight).

Table 10. Linear regression models with adiposity measures as the dependent variables and fatty acids ratios and proportions as the independent variables.

DepV	IndV	B	SE	SB	Pv	R ²	aR ²	Pm
BMI	Constant	-4.711	12.128		.700	.500	.450	<.001
	15:0+17:0	14.609	3.863	.514	.001			
	Unsat/Sat	26.047	9.618	.353	.011			
	Δ5	-1.022	.491	-.284	.046			
BMI z-sc	Constant	-1.745	.926		.069	.341	.299	.002
	Δ6	5.963	1.749	.501	.002			
	Elong ^b	10.843	4.299	.371	.017			
W/H	Constant	-.325	.256		.213	.483	.431	<.001
	15:0+17:0	.151	.049	.431	.004			
	UI	.007	.002	.493	.001			
	Δ5	-.016	.006	-.371	.013			
Weight	Constant	-45.581	38.697		.248	.404	.366	<.001
	15:0+17:0	50.439	12.124	.579	.000			
	Unsat/Sat	73.422	31.504	.325	.026			

DepV, dependent variable; IndV, independent variable; B, coefficient beta; SE, standard error; SB, stardartized B; Pv, levels of significance of the variable; R², squared correlation coefficient; aR², adjusted R²; Pm, level of significance of the model. BMI, body mass index; BMI z-scc, BMI z-score; W/H, waist-to-height ratio; FA, Fatty acid; n3, n 3 FA family; SFA, saturated FA; UFA, unsaturated FA; UI, unsaturation index. Δ5 desaturase, 20:4n6/20:3n6; Elong^b, 22:4n6/20:4n6; Δ 6 desaturase, 20:3n6/18:2n6 ratio; UI= Σ (fatty acids % * number of unsaturations).

When considering the FA proportions and ratios, the BMI is the adiposity measure which is more explained by the model (50 %) (Table 10).

A positive relation between the sum of odd numbered FA 15:0 and 17:0 with adiposity was identified. In our study the FA that is the main responsible by this relation is 15:0 since it was already included as an individual FA in the previous models. Considering that these two FA are usually related to the consumption of fat from ruminants, particularly in dairy products (131, 152), the increase in the proportion of these two FA in the RBC membrane, with increasing adiposity might be due to unbalanced diets with a high proportion of animal fat. High amounts of animal fat in diet, have already been linked with obesity. (121, 224) Thus, unbalanced nutritional habits appear to be present in our obese group.

The metabolism of ω 6 FA seem to have a close relation with obesity. The ratios used to estimate enzyme activities that appear to be altered with obesity all include ω 6 FA. In fact, two particular FA are present in all the ratios: AA and DGLA. The decrease in Δ 5 and the increase in Δ 6 and elongase activities result in decreased AA acid and increased DGLA and 22:4n6. This finding is different from other studies that found a decrease in Δ 6 activity with obesity and an increase in AA (84, 224). The AA is a FA closely linked to inflammation, due to its relation to eicosanoid synthesis. (215, 220) It is interesting that in our population, although inflammation is increased (as shown by increased CRP) the AA appear with diminished levels.

The majority of the studies point to a diminished level of RBC membrane unsaturation in obese individuals, mainly due to reduced n3 PUFA (86), although there is still controversy. (139) We found an increase in membrane FA unsaturation, as shown by the increase in UFA/SFA ratio with increasing BMI and weight, and by the positive relation between UI and the W/H ratio.

When using the FA ratios we found a particular division in the variables included in the models for BMI z-sc and weight. In agreement with the model for individual FA, BMI model appear to be unfolded in two, to originate BMI z-sc and weight models. BMI z-sc model included only variables involving ω 6 FA ratios, while weight models included the sum of 15:0 and 17:0 FA and a marker of membrane unsaturation, the unsaturation index.

In summary, our population showed an increase in the unsaturation of the RBC membrane with increasing obesity. This increase might be related with changes in the ω 6 FA metabolism. Despite the general increase in membrane unsaturation, there is an increase in some SFA. Some of these SFA are related with diet and suggest an increase in the consumption of animal fat. Different FA and FA derived ratios associated differently with BMI z-sc or with total body mass (weight). BMI z-sc was more closely associated with

changes in $\omega 6$ metabolism, while weight had a close relation with FA that are linked with animal fat ingestion. Interestingly, BMI appear to conjugate these two groups of influence.

7.5.2. Lipid Profile

Regarding the lipid profile the influence of SFA, PUFA and DMA is visible for many of the serum lipid variables (Tables 11 and 12).

Table 11. Linear regression models with lipid profile variables as the dependent variables and fatty acids as independent variables.

DepV	IndV	B	SE	SB	Pv	R ²	aR ²	Pm
TG	Constant	3.750	.109		<.001	.311	.289	.001
	15:0	1.148	.302	.557	.001			
LnChol	Constant	4.821	.213		<.001	.331	.287	.002
	22:0	1.181	.311	1.019	.001			
	22:4n6	-.458	.172	-.714	.012			
LnHDL	No variable entered the model							
LnLDL	Constant	3.474	.778		<.001	.403	.343	.001
	22:0	1.144	.428	.383	.012			
	18:1DMA	-2.132	.795	-.386	.012			
	22:5n3	.901	.406	.323	.034			
Apo A	Constant	180.123	16.793		<.001	.404	.366	<.001
	18:3n3	-240.645	62.463	-.535	.001			
	18:1DMA	-55.900	21.041	-.369	.012			
Apo B	Constant	47.714	20.056		.024	.291	.245	.005
	22:0	35.822	13.092	.414	.010			
	18:1DMA	-52.888	24.173	-.331	.036			
ApoA/ApoB	Constant	2.746	2.646		.308	.405	.346	.001
	16:0DMA	2.583	.910	.407	.008			
	20:0	-17.017	5.377	-.478	.004			
	18:1tsoma	-5.196	2.540	-.314	.050			
LnChol/HDL	Constant	.712	.267		.012	.301	.256	.004
	22:0	.515	.174	.443	.006			
	18:1DMA	-.659	.322	-.308	.049			
Lp (a)	No variable entered the model							

DepV, dependent variable; IndV, independent variable; B, coefficient beta; SE, standard error; SB, standardizd B; Pv, levels of significance of the variable; R², squared correlation coefficient; aR², adjusted R²; Pm, level of significance of the model. TG, triglycerides; Chol, total Cholesterol; HDL, high density lipoprotein Cholesterol; LDL, low density lipoprotein Cholesterol; Lp, lipoprotein; apo, apolipoprotein.

Obesity is known to be associated with a worsening lipid profile and diet is the main intervenient in this process. (123, 134) In fact, diet is closely linked to the type and amount of plasmatic lipids and many studies relate increased SFA consumption with a worse lipid profile. (127-128) This effect is particularly shown by the increased plasmatic concentrations of Chol and LDL in individuals whose diet include a high amount of those fats. Trans FA are also associated with dyslipidemia. (128) The RBC FA profile can be used as a marker of diet FA, but this should be considered carefully, as endogenous changes in FA and lipid metabolism in obesity also plays an important part. (237)

TG present a direct relationship with odd numbered FA, particularly with 15:0, which explains alone 31.1% of the TG values. The sum 15:0 + 17:0 is also linked to an increase in apo B, the main apolipoprotein of LDL. Lp (a) is known to be, mainly, genetically determined. In our study Lp (a) levels were directly related with these two FA in the linear regression model involving the FA ratios, despite presenting no model for individual FA analysis. Considering that these FA come from animal fat in diet (131, 152), its relation with the worsening of the lipid profile is noteworthy.

Another SFA that have an impact in the lipid profile is the FA 22:0, which is directly related with an increase in Chol, LDL, apo B and Chol/HDL, while 20:0 is linked with a decrease in Apo A/ Apo B ratio. The association between 22:0 and obesity is in agreement with its impact on lipid profile.

The DMAs are usually considered to have beneficial effects in health as anti-oxidants and PUFA reserve. (245-246) Actually, they are usually reduced in obesity and other diseases characterized by chronic inflammation and oxidative stress, such as cancer and Parkinson. (245-246) In our population DMA are linked to an improvement in the lipid profile, as total DMA was directly linked to increased apo A/apo B ratio, being 16:0 DMA the most influent as it is present as an explanatory variable even as an individual FA. The behavior of 18:1 DMA is interesting. It is related with the decrease in LDL and with both apo A and apo B. The influence of 18:1 DMA in apo A could lead us to think that it would be related to the decrease of HDL and consequently to a worsen lipid profile. Even so, the impact on apo B and LDL must be greater than in apo A since 18:1 DMA is also related with a decrease in Chol/HDL ratio.

Dyslipidemia is normally associated to increased inflammation. A link between them can be the presence of oxidized LDL particles (oxLDL) that take part in the formation of foam cells and atherogenesis. (184, 269) Besides that, circulating oxLDL leads to macrophage activation and consequent release of pro-inflammatory mediators. Also, the appearance of

anti-oxLDL antibodies further increase the inflammatory process. (184, 269) The relation of DMA with increased CRP concentrations and with a better lipid profile is, therefore, contradictory. LDL particles rich in plasmalogens are said to be more oxidable. (245-246) The relation between 18:1DMA with increased apo B may be the link between lipids and inflammation that explain this contradiction. A possible way to clarify this relation would be to identify which FA is present in the sn2 position of the plasmalogens species present in the RBC membrane.

Omega 3 FA appear as associated to worsening of lipid profile, while ω 6 appear to have the opposite role (Table 11). In fact, ω 6 22:4n6 is inversely related with Chol, while ω 3 DHA is linked to an increase in LDL, and ALA is associated with reduced apo A. This fact is in agreement to the regression models based on FA ratios, as DHA_{n3}/DPA_{n6} was inversely related with apo A concentration and n3/n6 PUFA ratio was inversely linked to apo A/apo B ratio.

The activity of Δ 5 desaturase seems to be inversely related to a decrease in HDL. The ratio used to estimate this enzyme activity is 20:4n6/20:3n6. Thus, the inverse association of the ratio with HDL could be linked to the accumulation of AA, having a deleterious impact in lipid metabolism. (228) Obesity is associated with reduced HDL levels. (3-4) In our population, however, a trend to smaller Δ 5 activity with increasing adiposity was found and is not in agreement with a decrease in HDL. Again, the association of FA ratios should be analyzed carefully.

The lower chain n3 PUFA seems to have a worst impact on lipid profile, when compared with longer PUFA, as DHA_{n3} and DPA_{n3}. In fact ALA is related to the reduction of apo A levels, while n3 product/precursor ratio ((22:5n3+22:6n3)/18:3n3)) is related to their increase.

The trans FA are normally related to a worst lipid profile. We found that the FA 18:1t was linked to a decrease in apo A/ apo B ratio. (128)

The relation between the saturated FA 22:0, 15:0 and 17:0 with growing adiposity is in agreement with their relation with worsening of the lipid profile. On the other hand, 22:4n6 FA appears to be related with an improvement in lipid profile although it is associated with increasing obesity.

Table 12. Linear regression models with lipid profile variables as the dependent variables and fatty acids ratios and proportions as the independent variables.

DepV	IndV	B	SE	SB	Pv	R ²	aR ²	Pm
TG	Constant	3.433	.180		<.001	.321	.300	<.001
	15:0+17:0	1.102	.283	.567	<.001			
LnChol	No variable entered the model							
LnHDL	Constant	4.297	.065		<.001	.134	.107	.033
	Δ5	-.021	.009	-.366	.033			
LnLDL	No variable entered the model							
Apo A	Constant	83.493	11.325		<.001	.291	.246	.005
	n3 Product/precursor ratio	1.093	.324	.548	.002			
	DHAn3/DPA _n 6	-2.286	.979	-.380	.026			
Apo B	Constant	37.560	7.871		<.001	.126	.098	.040
	15:0+17:0	26.553	12.387	.354	.040			
ApoA/ApoB	Constant	5.151	3.132		.110	.265	.218	.008
	n3/n6 PUFA	-31.784	11.292	-.436	.008			
	DMA	.798	.380	.325	.044			
LnChol/HDL	No variable entered the model							
Lp (a)	Constant	2.500	.473		<.001	.130	.102	.037
	15:0+17:0	1.626	.745	.360	.037			

DepV, dependent variable; IndV, independent variable; B, coefficient beta; SE, standard error; SB, standardized B; Pv, levels of significance of the variable; R², squared correlation coefficient; aR², adjusted R²; Pm, level of significance of the model. TG, triglycerides; Chol, total Cholesterol; HDL, high density lipoprotein Cholesterol; LDL, low density lipoprotein Cholesterol; Lp, lipoprotein; apo, apolipoprotein; FA, Fatty acid; n3, n 3 FA family; PUFA, polyunsaturated FA; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; Δ, delta desaturase; DMA, dimethyl acetyl FA derivative. n3 Product/precursor ratio, (20:5+22:5+22:6)/n3/18:3n3; Δ5 desaturase, 20:4n6/20:3n6.

7.5.3. Glucose metabolism

Table 13. Linear regression models with glucose homeostasis markers as the dependent variables and fatty acids as independent variables.

DepV	IndV	B	SE	SB	Pv	R ²	aR ²	Pm
LnGlycemia	Constant	4.710	.118		<.001	.583	.508	<.001
	17:0	-1.384	.306	-.605	<.001			
	20:0	.797	.241	.435	.003			
	16:1n9	-.644	.239	-.330	.012			
	20:3n6	.073	.024	.386	.005			
	18:2tsoma	-.178	.062	-.373	.008			
LnInsulin	Constant	1.561	.445		.001	.590	.533	<.001
	22:0	1.568	.342	.678	<.001			
	20:0	-5.071	1.306	-.593	.001			
	18:3n3	5.032	1.552	.397	.003			
	18:1tsoma	-1.228	.513	-.309	.023			
LnHOMA_{IR}	Constant	-1.314	.517		.017	.542	.479	<.001
	22:0	1.632	.388	.656	<.001			
	20:3n6	.291	.123	.307	.025			
	18:3n3	5.073	1.767	.372	.008			
	20:0	-3.367	1.489	-.366	.031			

DepV, dependent variable; IndV, independent variable; B, coefficient beta; SE, standard error; SB, stardartized B; Pv, levels of significance of the variable; R², squared correlation coefficient; aR², adjusted R²; Pm, level of significance of the model. HOMA_{IR}, homeostasis model assessment - insulin resistance.

In these models, it is notable the increased impairment of glycemic metabolism with the increase of PUFA, ALA and DGLA. On the other hand, the FA 17:0, 16:1n9 and 18:2t were related with reduced levels of circulating glucose (Table 13).

Obesity is characterized by an increase in insulin resistance and consequent unbalance of glucose metabolism (270). The positive relation between SFA and obesity has been associated with the impairment in glucose homeostasis. (138) In fact, in our study, we found a positive relation between insulin and the FA docosanoic (22:0). On the other hand, the SFA 20:0 have an interesting behavior, as it increases the circulating level of glucose but reduces insulinemia. These somehow opposing effects have a net balance of diminishing IR, as is shown by 20:0 FA negative relations with HOMA_{IR}. The FA 20:0 was already associated with an increase in CRP. The conjugation of these two relations is curious, since CRP is positively linked to HOMA_{IR}. (271)

The trans FA are usually associated with increased IR. (151) Conversely, in our study we found an improvement in glucose homeostasis with this FA class, as 18:1t and 18:2t were associated with reduced insulin and glucose plasmatic concentrations, respectively.

Although 17:0 FA has been associated with an increase in adiposity (together with 15:0 acid), and adiposity is linked to worsening of metabolic control, 17:0 FA appear to be related to a decrease in glucose levels.

Table 14. Linear regression models with glucose homeostasis markers as the dependent variables and fatty acids ratios and proportions as the independent variables.

DepV	IndV	B	SE	SB	Pv	R ²	aR ²	Pm
LnGlycemia	Constant	3.636	.370		<.001	.147	.120	.025
	UI	.006	.003	.383	.025			
LnInsulin	Constant	1.879	.257		<.001	.118	.090	.047
	Δ6	2.235	1.082	.343	.047			
LnHOMA_{IR}	Constant	-3.624	1.766		.049	.252	.204	.011
	LCPUFA	.119	.053	.349	.032			
	Δ6	2.448	1.090	.349	.032			

DepV, dependent variable; IndV, independent variable; B, coefficient beta; SE, standard error; SB, stardartized B; Pv, levels of significance of the variable; R², squared correlation coefficient; aR², adjusted R²; Pm, level of significance of the model. HOMA_{IR}, homeostasis model assessment - insulin resistance ; Δ 6 desaturase, 20:3n6/18:2n6 ratio; UI= Σ (fatty acids % * number of unsaturations).UI, unsaturation index.

A higher UI is usually associated with an increase in insulin sensibility. (83-84) Conversely, we found a positive association of UI with the levels of glucose, and an increase in HOMA_{IR} with increased LCPUFA, although the models did not account for the explanation of a large part of these variables fluctuation (Table 14).

Some studies have pointed out that increased levels of ω 3 PUFA (especially EPA and DHA) from supplementation or diet, although related to an improvement of lipid profile, are associated with worsening of glucose metabolism. (226, 230) This might be the reason why the precursor of EPA and DHA, ALA, is associated with increased levels of insulin and HOMA_{IR}. Furthermore, glucose control may not be restricted to ω 3 FA but ω 6 FA might also interfere. Actually, n6 FA DGLA is present in glucose metabolism models constructed with both individual FA and FA ratios and proportions models. Also, an increase in the activity of Δ6 desaturase, with consequent increased concentration of DGLA (based on the formula used), is related to increased levels of insulin and HOMA_{IR}.

Changes in the membrane FA composition can have important effects in insulin signaling, glucose transport and, thus, in IR. (237)

The FA DGLA is positively associated to adiposity, CRP and HOMA_{IR} and could be part of the link between these metabolic syndrome variables.

7.5.4. C-Reactive protein

Table 15. Linear regression models with CRP as the dependent variable and fatty acids as independent variables.

DepV	IndV	B	SE	SB	Pv	R ²	aR ²	Pm
LnCRP	Constant	-5.650	1.948		.007	.598	.542	<.001
	18:0 DMA	1.557	.382	.499	<.001			
	20:3n6	1.069	.358	.387	.006			
	20:0	9.087	3.381	.338	.012			
	22:6n3	-.718	.316	-.305	.031			

DepV, dependent variable; IndV, independent variable; B, coefficient beta; SE, standard error; SB, stardartized B; Pv, levels of significance of the variable; R², squared correlation coefficient; aR², adjusted R²; Pm, level of significance of the model. CRP, C-reactive protein; n3, n 3 fatty acid family; DMA, dimethyl acetyl fatty acid derivative.

Table 16. Linear regression models with CRP as the dependent variable and fatty acids ratios and proportions as the independent variables.

DepV	IndV	B	SE	SB	Pv	R ²	aR ²	Pm
LnCRP	Constant	-1.990	1.545		.207	.426	.389	<.001
	Δ5	-.388	.109	-.487	.001			
	DMA	.871	.253	.470	.002			

B, coefficient beta; SE, standard error; SB, stardartized B; Pv, levels of significance of the variable; R², squared correlation coefficient; aR², adjusted R²; Pm, level of significance of the model. CRP, C-reactive protein; n3, n 3 fatty acid family; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; DMA, dimethyl acetyl fatty acid derivative. Δ5 desaturase, 20:4n6/20:3n6

The model considering only individual FA explains 59.8% of CRP variation, while the model based on the FA ratios explains 42.6% (Tables 15 and 16).

CRP plasmatic concentration is increased in obesity. (8, 10) The association between inflammation and FA has been greatly explored and the synthesis of eicosanoids and

docosanoids appear to be an important link between these metabolic processes. (211, 237)

The analysis of the relation of CRP with the FA profile shows a positive relation between DGLA and inflammation. DGLA is associated with increased CRP and the action of $\Delta 5$ desaturase, that transforms DGLA in AA, appears to be inversely related with inflammation. Our results seem to be, somehow, in conflict with other studies that point DGLA as a FA with anti-inflammatory properties, while AA is usually increased in inflammation. (211, 215) The mechanisms behind the functions of these FA are, usually, exerted by their metabolic product, the eicosanoids. Eicosanoids from DGLA are considered to be less inflammatory or anti-inflammatory, when compared to AA eicosanoids. (101, 236) The positive association found between DGLA, obesity markers and inflammation are not likely to be casual.

On the other hand, a $\omega 3$ PUFA, DHA, appears to have the opposite impact, as it has been associated with a decrease in CRP. In fact, DHA is usually associated with anti-inflammatory properties. (185-187)

In our study, the increase of DMA fatty acid derivatives was associated with an increase in CRP, shown by the positive relation between 18:0 DMA and total DMA percentage with CRP. DMA are the result of the process of derivatization, to obtain FAME, on the vinyl ether bonds present in plasmalogens. (245-246) Plasmalogens are considered to be anti-inflammatory and to prevent oxidative stress. (245-246) In our study we found the opposite relation. Plasmalogens are also considered to function as a PUFA reserve, since these are the main FA present on their sn2 position. In this way, and depending on the PUFA, plasmalogens could act as an important supply of PUFA for eicosanoids synthesis and consequent inflammation. (245-246) Besides that, as they have the vinyl ether bond in the sn1 position and PUFA on the sn2 position, plasmalogens are also more easily oxidable. Some of the oxidation products could promote the increase in inflammation. (245-246)

There are some studies proposing that SFA are related with an increase in inflammation. (83-84) The FA 20:0 had a positive impact in the levels of CRP. Interestingly, the FA 22:0 and 15:0 that are associated with obesity were not related with CRP.

8. Conclusions

Obesity is characterized by several changes in the body functioning. Some of these changes are associated to a metabolic condition called “metabolic syndrome”. Metabolic syndrome is a cluster of CVD risk factors, including dyslipidemia, insulin resistance, inflammation and obesity itself. (128) Although still young, our obese population already presented significant changes in all these variables, when compared to their lean counterparts.

In our population the obese group presented with a worst lipid profile, increased IR and inflammation. It is remarkable the presence of metabolic changes in such young ages. These changes tend to perpetuate themselves through adulthood and are accompanied by increased risk for many diseases. In fact, childhood obesity is associated with the development of CVD and T2DM in adulthood, besides increasing the probability of maintaining obesity through the adult life. (15-16)

The RBC, as a cell with limited repairing capacity, appears as a potential marker to study the impact of the obesity related metabolic changes. Besides that, as the RBC lifespan is of about 120 days, the RBC may transmit information about what occurred in the organism during that period being, for example, an interesting toll to follow therapeutic approaches. (237) The differences in erythrogram and in markers of erythrocyte damage were not observed in our population. Nevertheless, it seems to be present in the obese individuals a somehow heterogeneous RBCs population, mixing older damaged cells with younger ones. RBC could be injured by increased oxidative stress and inflammatory mediators, common in obesity, leading to a shorter lifespan of the cell and, consequently, to an increased erythropoietic stimulus needed to replace the senescent RBC population. Furthermore, there is the need to supply blood to the growing adipose tissue that further stimulates erythropoiesis. (11-12, 264)

The analysis of the FA profile revealed some differences between the BMI percentile groups. The FA 22:0 was significantly higher in obese, what is in agreement with many studies that point SFA to be increased in obesity. Also, there was a trend for increased 15:0, 17:0 and 20:0. The unsaturation of the membrane is usually said to be diminished in obese individuals. Nevertheless, we found an increase in PUFA 18:3n3, 20:3n6 and 22:2n6 with increasing BMI percentile. The increase in n6 PUFA has been proposed as a body mechanism to compensate for the decrease in the membrane unsaturation, caused, mainly, by a reduction in n3 FA ingestion.

The linear regression analysis revealed interesting associations between FA and the other variables studied. Some FA, namely 20:0, 22:0, 15:0, 17:0, 18:3n3, 20:3n6 and 22:2n6 seem to be particularly important in these as they are associated with worsening of the metabolic control and with increased CVD risk markers.

An increase in inflammation was associated with increased levels of 20:0 and 20:3n6. The FA 20:3n6, DGLA, can be transformed in eicosanoids and function as inflammatory mediators. Despite that, the majority of the studies point 20:3n6 eicosanoid products to be either anti-inflammatory or mildly inflammatory. Actually, 20:4n6 (AA) and eicosanoids are said to present the stronger pro-inflammatory properties. (211, 237) In obesity, a change in FA metabolism could be present towards the decrease in AA and an increase in 20:3n6, could result from variations in enzymes activities. Differences in diet could also play a role.

DHA, 22:6n3, is usually associated with anti-inflammatory properties and has a particular importance in the CNS. (170-171) It was the only FA associated with a decrease in CRP levels and, as a result, in inflammation.

DMA FA derivatives are the products of plasmalogens in the derivatization process. Plasmalogens are usually accepted as anti-oxidants and anti-inflammatory, due to its particular vinyl ether bond in position sn1 and to the PUFA usually present in position sn2. (245-246) However, we found a positive relation between DMA FA derivatives and CRP. Some studies reported that the presence of increased oxidative stress, as occurs in obesity, could lead to the oxidation of plasmalogens and production of deleterious pro-inflammatory products of lipid peroxidation that would further propagate inflammation. (245-246)

Glucose homeostasis is disturbed in obese individuals. SFA and n3 PUFA have been related to worsening of glucose control. (83-84) In agreement, we found 22:0 and 18:3n3 to be associated with an increase in IR. Despite that, another SFA, eicosanoic acid (20:0), was associated with a decrease in IR. DGLA, 20:3n6, was also associated with increased IR and could be part of the link between inflammation and IR. The unsaturation level of the membrane was positively associated with IR, contrarily to what is generally referred in obesity. (83-84) More than the absolute unsaturation of the membrane it is important to focus on which FA are contributing to this increase, since n3 FA are associated with increased IR. Besides, there is a close link between FA, inflammation and IR. Changes in membrane FA composition may influence insulin mediated glucose transport, by altering

glucose transporters conformation. Furthermore, some of the FA are important 2nd messengers in insulin signaling.

Regarding the serum lipid profile, most of the mentioned FA are related to its worsening. The increase in odd numbered 15:0 and 17:0 acids was related with a increase in TG. The other FA impact appears to be towards a worsening of the Chol balance (20:0, 22:0 and 18:3n3), HDL and LDL. An increased ingestion of SFA in diet have been associated with risk/changes in the lipid profile, especially, in Chol. On the other hand, n3 FA are usually associated with improvement in dyslipidemia, despite a negative impact on glucose metabolic control. (230) In our population we found, indeed, the expected association with SFA, but not with n3 FA. In fact, ALA (18:3n3) was negatively associated with apo A, and DPAn3 (22:5n3) was directly related to LDL. On the other hand, n6 FAs appear to have a more positive relation with serum lipids, as 22:4n6 were related to a decrease in Chol. The DMA FA derivatives are associated with an increase in apo A/apo B and a decrease in Chol/HDL ratios and, thus, an improvement in lipid profiles.

In resume, obese individuals present increased markers of CVD when compared to their lean counterparts, although no were visible changes were observed in erythrogram and erythrocyte damage markers. Concerning the FA membrane profile, specific FA were associated with metabolic syndrome features (IR, inflammation and dyslipidemia). Further studies are needed to clarify the associations between membrane FA, RBC injury markers and CVD risk markers. An increase in the number of individuals studied could also help to clarify some trends. Regarding the membrane analysis itself some interesting approaches could be made as to separate and analyze the different PL and CE and their associated FA, and to perform a separate study of inner and outer membrane sheets. Furthermore, optimization of the separation technique and consequent identification of FA would be crucial, as in this study we could not evaluate important FA, e.g. EPA (20:5n3) and GLA (18:3n6), due to technical limitations.

9. References

1. Red Blood Cells. [cited 2011 04/10/2011]; Available from: <http://www-lsp.ujf-grenoble.fr/equipe/dyfcom/quilliet/ShapeComp.htm>.
2. OECD OFEC-OAD-. Health at a Glance 2009 - OECD Indicators: OECD Publishing 2009. p. 43-58.
3. Nascimento H, Rocha S, Rego C, Mansilha HF, Quintanilha A, Santos-Silva A, et al. Leukocyte Count versus C-Reactive Protein Levels in Obese Portuguese Patients Aged 6-12 Years Old. *Open Biochem J*. 4:72-6.
4. Nascimento H, Silva L, Lourenco P, Weinfurterova R, Castro E, Rego C, et al. Lipid profile in Portuguese obese children and adolescents: interaction of apolipoprotein E polymorphism with adiponectin levels. *Arch Pediatr Adolesc Med*. 2009;163(11):1030-6.
5. Obesity-related disease. [cited 2011 04/10/2011]; Available from: <http://drugster.info/medic/term/obesity-related-disease/>.
6. Pilz S, Horejsi R, Moller R, Almer G, Scharnagl H, Stojakovic T, et al. Early atherosclerosis in obese juveniles is associated with low serum levels of adiponectin. *The Journal of clinical endocrinology and metabolism*. 2005;90(8):4792-6.
7. Tsigos C, Kyrou I, Chala E, Tsapogas P, Stavridis JC, Raptis SA, et al. Circulating tumor necrosis factor alpha concentrations are higher in abdominal versus peripheral obesity. *Metabolism: clinical and experimental*. 1999;48(10):1332-5.
8. Wozniak SE, Gee LL, Wachtel MS, Frezza EE. Adipose Tissue: The New Endocrine Organ? A Review Article. *Digestive diseases and sciences*. 2008.
9. [cited 2011 04/10/2011]; Available from: http://www.medscape.com/viewarticle/745306_7.
10. Alexander CM, Landsman PB, Grundy SM. The influence of age and body mass index on the metabolic syndrome and its components. *Diabetes, obesity & metabolism*. 2008;10(3):246-50.
11. Frayn KN, Karpe F, Fielding BA, Macdonald IA, Coppack SW. Integrative physiology of human adipose tissue. *Int J Obes Relat Metab Disord*. 2003;27(8):875-88.
12. Lolmede K, Durand de Saint Front V, Galitzky J, Lafontan M, Bouloumie A. Effects of hypoxia on the expression of proangiogenic factors in differentiated 3T3-F442A adipocytes. *Int J Obes Relat Metab Disord*. 2003;27(10):1187-95.
13. Ignarro LJ, Balestrieri ML, Napoli C. Nutrition, physical activity, and cardiovascular disease: an update. *Cardiovascular research*. 2007;73(2):326-40.
14. Smith JK, Dykes R, Douglas JE, Krishnaswamy G, Berk S. Long-term exercise and atherogenic activity of blood mononuclear cells in persons at risk of developing ischemic heart disease. *Jama*. 1999;281(18):1722-7.
15. COSI. European Childhood Obesity Surveillance Initiative - In: Saúde Md, editor. 2009.
16. Padez C, Fernandes T, Mourao I, Moreira P, Rosado V. Prevalence of overweight and obesity in 7-9-year-old Portuguese children: trends in body mass index from 1970-2002. *Am J Hum Biol*. 2004;16(6):670-8.
17. Rocha S. Biological aspects underlying the clinical outcome of Hereditary Spherocytosis. Porto: Porto; 2010.
18. Delaunay J. The molecular basis of hereditary red cell membrane disorders. *Blood Rev*. 2007;21(1):1-20. Epub 2006/05/30.
19. Brovelli A, Castellana MA, Minetti G, Piccinini G, Seppi C, De Renzis MR, et al. Conformational changes and oxidation of membrane proteins in senescent human erythrocytes. *Advances in experimental medicine and biology*. 1991;307:59-73.
20. Lutz H. Erythrocyte clearance blood cell biochemistry. In: Harris J, editor. *Erythroid cells*. New York: New York: Plenum; 1990. p. 81-120.
21. Santos-Silva A, Rebelo MI, Castro EM, Belo L, Guerra A, Rego C, et al. Leukocyte activation, erythrocyte damage, lipid profile and oxidative stress imposed by high competition physical exercise in adolescents. *Clinica chimica acta; international journal of clinical chemistry*. 2001;306(1-2):119-26.
22. Weiss DJ, Aird B, Murtaugh MP. Neutrophil-induced immunoglobulin binding to erythrocytes involves proteolytic and oxidative injury. *Journal of leukocyte biology*. 1992;51(1):19-23.
23. Ciesla B. Normochromic Anemias, Biochemical and Membrane Disorders, and Miscellaneous Red Cell Disorders. In: Ciesla B, editor. *Hematology in Practice*. Philadelphia: F. A. Davis Company 2007. p. 97-112.
24. Gallagher PG. Red cell membrane abnormalities. In: Robert J. Arceci IMH, Owen P. Smith, editor. *Pediatric Hematology*. Third ed. Massachusetts: Blackwell Publishing Ltd; 2006. p. 255-80.

25. Yawata Y. Composition of Normal Red Cell Membranes. In: Yawata Y, editor. *Cell Membrane: The Red Blood Cell as a Model*. Weinheim: WILEY-VCH Verlag GmbH & Co; 2003. p. 27-46.
26. Red Blood Cell. [cited 2011 04/10/2011]; Available from: <http://rbclab.com/Pages/500/520/520.html>
27. Pinto E, Ramos E, Severo M, Casal S, Dos Santos Silva I, Lopes C, et al. Measurement of dietary intake of fatty acids in pregnant women: comparison of self-reported intakes with adipose tissue levels. *Ann Epidemiol*. 2010;20(8):599-603. Epub 2010/07/09.
28. Yawata Y. Skeletal Proteins. In: Yawata Y, editor. *Cell Membrane: The Red Blood Cell as a Model*. Weinheim: WILEY-VCH Verlag GmbH & Co; 2003. p. 61-80
29. Yawata Y. Integral Proteins. In: Yawata Y, editor. *Cell Membrane: The Red Blood Cell as a Model*. Weinheim: WILEY-VCH Verlag GmbH & Co; 2003. p. 81-113.
30. Yawata Y. Anchoring Proteins. In: Yawata Y, editor. *Cell Membrane: The Red Blood Cell as a Model*. Weinheim: WILEY-VCH Verlag GmbH & Co; 2003. p. 115-32.
31. Tse WT, Lux SE. Red blood cell membrane disorders. *Br J Haematol*. 1999;104(1):2-13. Epub 1999/02/23.
32. Mohandas N, Gallagher PG. Red cell membrane: past, present, and future. *Blood*. 2008;112(10):3939-48. Epub 2008/11/08.
33. An X, Mohandas N. Disorders of red cell membrane. *Br J Haematol*. 2008;141(3):367-75. Epub 2008/03/18.
34. Iolascon A, Perrotta S, Stewart GW. Red blood cell membrane defects. *Rev Clin Exp Hematol*. 2003;7(1):22-56. Epub 2003/12/25.
35. Perrotta S, Gallagher PG, Mohandas N. Hereditary spherocytosis. *Lancet*. 2008;372(9647):1411-26. Epub 2008/10/23.
36. Patrick G Gallagher BG. Hereditary Spherocytosis, Hereditary Elliptocytosis, and Other Disorders Associated with Abnormalities of the Erythrocyte Membrane. In: George M. Rodgers FP, Bertil Glader, Daniel A. Arber, Robert T. Means, editor. *Wintrobe's Clinical Hematology*. 12 ed. Philadelphia: Lippincott Williams & Wilkins; 2009. p. 912-33.
37. Peters LL, Lux SE. Ankyrins: structure and function in normal cells and hereditary spherocytes. *Semin Hematol*. 1993;30(2):85-118. Epub 1993/04/01.
38. Su Y, Ding Y, Jiang M, Jiang W, Hu X, Zhang Z. Associations of protein 4.2 with band 3 and ankyrin. *Mol Cell Biochem*. 2006;289(1-2):159-66. Epub 2006/05/24.
39. Quizlet. [updated 25/10/2010; cited 2011 04/10/2011]; Available from: <http://quizlet.com/3539071/hematology-collective-flash-cards/>.
40. Hanspal M, Palek J. Biogenesis of normal and abnormal red blood cell membrane skeleton. *Semin Hematol*. 1992;29(4):305-19. Epub 1992/10/01.
41. Hanspal M, Prchal JT, Palek J. Biogenesis of erythrocyte membrane skeleton in health and disease. *Stem Cells*. 1993;11 Suppl 1:8-12. Epub 1993/05/01.
42. Yawata Y. Membrane Morphogenesis in Erythroid Cells. In: Yawata Y, editor. *Cell Membrane: The Red Blood Cell as a Model*. Weinheim: WILEY-VCH Verlag GmbH & Co; 2003. p. 133-54.
43. Kern WF. Metabolism and Function of Erythrocytes and the Metabolism of Iron. In: Kern WF, editor. *PDQ Hematology*. Ontario: BC Decker Inc; 2002. p. 25-33.
44. Telen MJ. The Mature Erythrocyte. In: George M. Rodgers FP, Bertil Glader, Daniel A. Arber, Robert T. Means, editor. *Wintrobe's Clinical Hematology*. 12 ed. Philadelphia: Lippincott Williams & Wilkins; 2009. p. 127-55.
45. Mallozzi C, Di Stasi AM, Minetti M. Free radicals induce reversible membrane-cytoplasm translocation of glyceraldehyde-3-phosphate dehydrogenase in human erythrocytes. *Archives of biochemistry and biophysics*. 1995;321(2):345-52. Epub 1995/08/20.
46. Rogalski AA, Steck TL, Waseem A. Association of glyceraldehyde-3-phosphate dehydrogenase with the plasma membrane of the intact human red blood cell. *The Journal of biological chemistry*. 1989;264(11):6438-46. Epub 1989/04/15.
47. Tsai AC, Sandretto A, Chung YC. Dieting is more effective in reducing weight but exercise is more effective in reducing fat during the early phase of a weight-reducing program in healthy humans. *The Journal of nutritional biochemistry*. 2003;14(9):541-9.
48. Schrier SL. Organization of enzymes in human erythrocyte membranes. *Am J Physiol*. 1966;210(1):139-45. Epub 1966/01/01.
49. Beutler E. How do red cell enzymes age? A new perspective. *Br J Haematol*. 1985;61(3):377-84.
50. Beutler E, Hartman G. Age-related red cell enzymes in children with transient erythroblastopenia of childhood and with hemolytic anemia. *Pediatr Res*. 1985;19(1):44-7. Epub 1985/01/01.

51. Suzuki T, Dale GL. Senescent erythrocytes: isolation of in vivo aged cells and their biochemical characteristics. *Proceedings of the National Academy of Sciences of the United States of America*. 1988;85(5):1647-51. Epub 1988/03/01.
52. Boas FE, Forman L, Beutler E. Phosphatidylserine exposure and red cell viability in red cell aging and in hemolytic anemia. *Proceedings of the National Academy of Sciences of the United States of America*. 1998;95(6):3077-81. Epub 1998/04/18.
53. de Jong K, Larkin SK, Eber S, Franck PF, Roelofsen B, Kuypers FA. Hereditary spherocytosis and elliptocytosis erythrocytes show a normal transbilayer phospholipid distribution. *Blood*. 1999;94(1):319-25. Epub 1999/06/25.
54. Bosman GJ, Lasonder E, Luten M, Roerdinkholder-Stoelwinder B, Novotny VM, Bos H, et al. The proteome of red cell membranes and vesicles during storage in blood bank conditions. *Transfusion*. 2008;48(5):827-35. Epub 2008/03/19.
55. Bosman GJ, Willekens FL, Werre JM. Erythrocyte aging: a more than superficial resemblance to apoptosis? *Cell Physiol Biochem*. 2005;16(1-3):1-8. Epub 2005/08/27.
56. Arese P, Turrini F, Schwarzer E. Band 3/complement-mediated recognition and removal of normally senescent and pathological human erythrocytes. *Cell Physiol Biochem*. 2005;16(4-6):133-46. Epub 2005/11/23.
57. Pantaleo A, Giribaldi G, Mannu F, Arese P, Turrini F. Naturally occurring anti-band 3 antibodies and red blood cell removal under physiological and pathological conditions. *Autoimmun Rev*. 2008;7(6):457-62. Epub 2008/06/19.
58. Rettig MP, Low PS, Gimm JA, Mohandas N, Wang J, Christian JA. Evaluation of biochemical changes during in vivo erythrocyte senescence in the dog. *Blood*. 1999;93(1):376-84. Epub 1998/12/24.
59. Turrini F, Giribaldi G, Carta F, Mannu F, Arese P. Mechanisms of band 3 oxidation and clustering in the phagocytosis of Plasmodium falciparum-infected erythrocytes. *Redox Rep*. 2003;8(5):300-3. Epub 2004/02/14.
60. Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. *Int J Biochem Cell Biol*. 2007;39(1):44-84. Epub 2006/09/19.
61. Valko M, Rhodes CJ, Moncol J, Izakovic M, Mazur M. Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chem Biol Interact*. 2006;160(1):1-40. Epub 2006/01/25.
62. Glader B. Destruction of Erythrocytes. In: George M, Rodgers FP, Bertil Glader, Daniel A. Arber, Robert T. Means, editor. *Wintrobe's Clinical Hematology*. 12 ed. Philadelphia: Lippincott Williams & Wilkins; 2009. p. 157-69.
63. Arashiki N, Otsuka Y, Ito D, Yang M, Komatsu T, Sato K, et al. The covalent modification of spectrin in red cell membranes by the lipid peroxidation product 4-hydroxy-2-nonenal. *Biochemical and biophysical research communications*. 2010;391(3):1543-7. Epub 2009/12/29.
64. Best B. Mechanisms of Aging. [cited 2011 11/10/2011]; Available from: <http://www.benbest.com/lifeext/aging.html>.
65. Santos-Silva A, Castro EM, Teixeira NA, Guerra FC, Quintanilha A. Erythrocyte membrane band 3 profile imposed by cellular aging, by activated neutrophils and by neutrophilic elastase. *Clinica chimica acta; international journal of clinical chemistry*. 1998;275(2):185-96.
66. Low FM, Hampton MB, Winterbourn CC. Peroxiredoxin 2 and peroxide metabolism in the erythrocyte. *Antioxid Redox Signal*. 2008;10(9):1621-30. Epub 2008/05/16.
67. Wood ZA, Schroder E, Robin Harris J, Poole LB. Structure, mechanism and regulation of peroxiredoxins. *Trends Biochem Sci*. 2003;28(1):32-40. Epub 2003/01/09.
68. Rhee SG, Chae HZ, Kim K. Peroxiredoxins: a historical overview and speculative preview of novel mechanisms and emerging concepts in cell signaling. *Free Radic Biol Med*. 2005;38(12):1543-52. Epub 2005/05/27.
69. Nagababu E, Chrest FJ, Rifkind JM. Hydrogen-peroxide-induced heme degradation in red blood cells: the protective roles of catalase and glutathione peroxidase. *Biochimica et biophysica acta*. 2003;1620(1-3):211-7. Epub 2003/02/22.
70. Kay MM. Role of physiologic autoantibody in the removal of senescent human red cells. *Journal of supramolecular structure*. 1978;9(4):555-67.
71. Kay MM. Localization of senescent cell antigen on band 3. *Proceedings of the National Academy of Sciences of the United States of America*. 1984;81(18):5753-7.
72. Kay MM, Goodman SR, Sorensen K, Whitfield CF, Wong P, Zaki L, et al. Senescent cell antigen is immunologically related to band 3. *Proceedings of the National Academy of Sciences of the United States of America*. 1983;80(6):1631-5.
73. Lutz HU. Naturally occurring anti-band 3 antibodies. *Transfusion medicine reviews*. 1992;6(3):201-11.

74. Lutz HU, Bussolino F, Flepp R, Fasler S, Stammers P, Kazatchkine MD, et al. Naturally occurring anti-band-3 antibodies and complement together mediate phagocytosis of oxidatively stressed human erythrocytes. *Proceedings of the National Academy of Sciences of the United States of America*. 1987;84(21):7368-72.
75. Ando K, Kikugawa K, Beppu M. Involvement of sialylated poly-N-acetyllactosaminyl sugar chains of band 3 glycoprotein on senescent erythrocytes in anti-band 3 autoantibody binding. *The Journal of biological chemistry*. 1994;269(30):19394-8. Epub 1994/07/29.
76. Beppu M, Mizukami A, Ando K, Kikugawa K. Antigenic determinants of senescent antigen of human erythrocytes are located in sialylated carbohydrate chains of Band 3 glycoprotein. *The Journal of biological chemistry*. 1992;267(21):14691-6. Epub 1992/07/25.
77. Kay MM, Lin FB. Molecular mapping of the active site of an aging antigen: senescent cell antigen requires lysine(s) for antigenicity and is located on an anion-binding segment of band 3 membrane transport protein. *Gerontology*. 1990;36(5-6):293-305. Epub 1990/01/01.
78. Kay MM, Marchalonis JJ, Hughes J, Watanabe K, Schluter SF. Definition of a physiologic aging autoantigen by using synthetic peptides of membrane protein band 3: localization of the active antigenic sites. *Proceedings of the National Academy of Sciences of the United States of America*. 1990;87(15):5734-8. Epub 1990/08/01.
79. Santos-Silva A, Rebelo I, Castro E, Belo L, Catarino C, Monteiro I, et al. Erythrocyte damage and leukocyte activation in ischemic stroke. *Clinica chimica acta; international journal of clinical chemistry*. 2002;320(1-2):29-35.
80. Ciesla B. Hemoglobin Function and Principles of Hemolysis. In: Ciesla B, editor. *Hematology in Practice*. Philadelphia: F. A. Davis Company 2007. p. 51-64.
81. Santos-Silva A, Castro EM, Teixeira NA, Guerra FC, Quintanilha A. Altered erythrocyte membrane band 3 profile as a marker in patients at risk for cardiovascular disease. *Atherosclerosis*. 1995;116(2):199-209.
82. Min Y, Lowy C, Ghebremeskel K, Thomas B, Offley-Shore B, Crawford M. Unfavorable effect of type 1 and type 2 diabetes on maternal and fetal essential fatty acid status: a potential marker of fetal insulin resistance. *The American journal of clinical nutrition*. 2005;82(6):1162-8.
83. Cazzola R, Rondanelli M, Russo-Volpe S, Ferrari E, Cestaro B. Decreased membrane fluidity and altered susceptibility to peroxidation and lipid composition in overweight and obese female erythrocytes. *Journal of lipid research*. 2004;45(10):1846-51.
84. Elizondo A, Araya J, Rodrigo R, Signorini C, Sgherri C, Comporti M, et al. Effects of weight loss on liver and erythrocyte polyunsaturated fatty acid pattern and oxidative stress status in obese patients with non-alcoholic fatty liver disease. *Biological research*. 2008;41(1):59-68.
85. Sola E, Vaya A, Santaolalia ML, Hernandez-Mijares A, Reganon E, Vila V, et al. Erythrocyte deformability in obesity measured by ektacytometric techniques. *Clinical hemorheology and microcirculation*. 2007;37(3):219-27.
86. Pasanisi F, Contaldo F, de Simone G, Mancini M. Benefits of sustained moderate weight loss in obesity. *Nutr Metab Cardiovasc Dis*. 2001;11(6):401-6.
87. Wolf AM, Beisiegel U. The effect of loss of excess weight on the metabolic risk factors after bariatric surgery in morbidly and super-obese patients. *Obesity surgery*. 2007;17(7):910-9.
88. The Nomenclature of Lipids. *European Journal of Biochemistry*. 1977;79(1):11-21.
89. Fatty acid structure. 2010 [updated 28/04/2010; cited 2011 11/10/2011]; Available from: http://pudsandlosers.blogspot.com/2010_04_25_archive.html.
90. IUPAC. IUPAC Gold Book. [cited 2011 04-10-2011]; Available from: <http://goldbook.iupac.org/index.html>.
91. Devlin TM. Lipid Metabolism I: Utilization and storage of energy in lipid form. *Textbook of biochemistry: With clinical correlations*. 5 ed. New York: Wiley-Liss; 2002.
92. Jeremy M Berg JLT, Lubert Stryer. *Biochemistry*. In: 2002, editor. New York: W H Freeman; 2002.
93. Beermann C, Jelinek J, Reinecker T, Hauenschild A, Boehm G, Klor HU. Short term effects of dietary medium-chain fatty acids and n-3 long-chain polyunsaturated fatty acids on the fat metabolism of healthy volunteers. *Lipids Health Dis*. 2003;2:10. Epub 2003/11/19.
94. Types of fatty acids. [cited 2011 11/10/2011]; Available from: <http://www.aafp.org/afp/2009/0815/afp20090815p345-f1.gif>.
95. David J. Anneken SB, Ralf Christoph, Georg Fieg, Udo Steinberner, Alfred Westfechtel. *Fatty Acids*. Ullmann's Encyclopedia of Industrial Chemistry. 2006.
96. Trans and cis double bonds. 2010 [updated 28/01/2010; cited 2011 11/10/2011]; Available from: <http://fitnessblackbook.com/diet-tips/the-four-types-of-fat-sorting-out-the-confusion-of-dietary-fats/>.

97. CDD CfDaD-. Conformations of nutritionally important fatty acids. [cited 2011 11/10/2011]; Available from: <http://www.cdd-tum.de/index.php?id=58>.
98. 2011 [updated 02/10/2011; cited 2011 11/10/2011]; Available from: http://en.wikipedia.org/wiki/Alpha-Linolenic_acid.
99. Fatty acids melting point. [updated 29/08/2010; cited 2011 11/10/2011]; Available from: <http://redzuannorazlan.blogspot.com/2010/08/bbc1-k22-lipid.html>.
100. Klesney JRaSP. Nomenclature of Organic Chemistry. 4 ed. Oxford: Pergamon Press; 1979.
101. Soberman RJ, Christmas P. The organization and consequences of eicosanoid signaling. *The Journal of clinical investigation*. 2003;111(8):1107-13. Epub 2003/04/17.
102. Ferre P, Foufelle F. SREBP-1c transcription factor and lipid homeostasis: clinical perspective. *Horm Res*. 2007;68(2):72-82. Epub 2007/03/09.
103. Christie WW. Fatty acids: straight-chain saturated. AOCs (American Oil Chemists' Society); [cited 2011 04/10/2011]; Available from: http://lipidlibrary.aocs.org/lipids/fa_sat/index.htm.
104. Bender D. Do we need to synthesise fatty acids? [updated 02/03/2010; cited 2011 11/10/2011]; Available from: <http://david-bender.purplecloud.net/metonline/lipids/fasynth/fasyn16.htm>.
105. Reginald H. Garrett CMG. Lipid Biosynthesis. *Biochemistry*. Harcourt: Harcourt College Publishers; 1999. p. 802-51.
106. Institute JJD-RRP. Fatty Acid Synthesis. [cited 2011 04/10/2011]; Available from: <http://rpi.edu/dept/bcbp/molbiochem/MBWeb/mb2/part1/fasynthesis.htm>.
107. Donald Voet JGV, Charlotte W. Pratt. Fatty acids Biosynthesis. *Fundamentals of Biochemistry: Life at the Molecular Level*. 3 ed. San Francisco: John Wiley & Sons; 2008. p. 930-8.
108. Pelley JW. Elsevier's Integrated Biochemistry. Maryland Heights: Mosby Elsevier; 2007.
109. Leibundgut M, Maier T, Jenni S, Ban N. The multienzyme architecture of eukaryotic fatty acid synthases. *Curr Opin Struct Biol*. 2008;18(6):714-25. Epub 2008/10/25.
110. Stephen R. Bolsover JSH, Elizabeth A. Shephard, Hugh A. White, Claudia G. Wiedeman. *Cell Biology: A Short Course*. Hoboken: John Wiley & Sons; 2004.
111. Wong K. Making fat-proof mice. 2000 [updated 29/10/2000; cited 2011 04/10/2011]; Available from: <http://www.scientificamerican.com/article.cfm?id=making-fat-proof-mice>.
112. Zechner R, Strauss JG, Haemmerle G, Lass A, Zimmermann R. Lipolysis: pathway under construction. *Curr Opin Lipidol*. 2005;16(3):333-40. Epub 2005/05/14.
113. De Vivo DC, Bohan TP, Coulter DL, Dreifuss FE, Greenwood RS, Nordli DR, Jr., et al. L-carnitine supplementation in childhood epilepsy: current perspectives. *Epilepsia*. 1998;39(11):1216-25. Epub 1998/11/20.
114. Kim KH. Regulation of mammalian acetyl-coenzyme A carboxylase. *Annu Rev Nutr*. 1997;17:77-99. Epub 1997/01/01.
115. Reginald H. Garrett CMG. Fatty Acid Catabolism. *Biochemistry*. Harcourt: Harcourt College Publishers; 1999. p. 775-801.
116. Mary K. Campbell SOF. *Biochemistry*. 5 ed. Andover: Cengage Learning; 2006. 579 p.
117. (USDA) USDoA. Reports by Single Nutrients [updated 09/28/2011; cited 2011 04/10/2011]; Available from: <http://www.ars.usda.gov/Services/docs.htm?docid=22114>.
118. A.J. German JZ. The Role of Nutrition. [updated 01/01/2008; cited 2011 11/10/2011]; Available from: <http://www.epi4dogs.com/theroleofnutrition.htm>.
119. Lipoprotein diagram. [cited 2011 11/10/2011]; Available from: <http://apbrwww5.apsu.edu/thompsonj/Anatomy%20&%20Physiology/2020/2020%20Exam%20Reviews/Exam%204/lipoprotein%20diagram03.bmp>
120. Danaei G, Ding EL, Mozaffarian D, Taylor B, Rehm J, Murray CJ, et al. The preventable causes of death in the United States: comparative risk assessment of dietary, lifestyle, and metabolic risk factors. *PLoS Med*. 2009;6(4):e1000058. Epub 2009/04/29.
121. Mozaffarian D, Clarke R. Quantitative effects on cardiovascular risk factors and coronary heart disease risk of replacing partially hydrogenated vegetable oils with other fats and oils. *Eur J Clin Nutr*. 2009;63 Suppl 2:S22-33. Epub 2009/05/09.
122. Mozaffarian D, Micha R, Wallace S. Effects on coronary heart disease of increasing polyunsaturated fat in place of saturated fat: a systematic review and meta-analysis of randomized controlled trials. *PLoS Med*. 2010;7(3):e1000252. Epub 2010/03/31.
123. Mente A, de Koning L, Shannon HS, Anand SS. A systematic review of the evidence supporting a causal link between dietary factors and coronary heart disease. *Archives of internal medicine*. 2009;169(7):659-69. Epub 2009/04/15.
124. Siri-Tarino PW, Sun Q, Hu FB, Krauss RM. Meta-analysis of prospective cohort studies evaluating the association of saturated fat with cardiovascular disease. *The American journal of clinical nutrition*. 2010;91(3):535-46. Epub 2010/01/15.

125. Jebb SA, Lovegrove JA, Griffin BA, Frost GS, Moore CS, Chatfield MD, et al. Effect of changing the amount and type of fat and carbohydrate on insulin sensitivity and cardiovascular risk: the RISCK (Reading, Imperial, Surrey, Cambridge, and Kings) trial. *The American journal of clinical nutrition*. 2010;92(4):748-58. Epub 2010/08/27.
126. Edem DO. Palm oil: biochemical, physiological, nutritional, hematological, and toxicological aspects: a review. *Plant Foods Hum Nutr*. 2002;57(3-4):319-41. Epub 2003/02/27.
127. Clarke R, Frost C, Collins R, Appleby P, Peto R. Dietary lipids and blood cholesterol: quantitative meta-analysis of metabolic ward studies. *BMJ*. 1997;314(7074):112-7. Epub 1997/01/11.
128. Ascherio A, Katan MB, Zock PL, Stampfer MJ, Willett WC. Trans fatty acids and coronary heart disease. *The New England journal of medicine*. 1999;340(25):1994-8. Epub 1999/06/24.
129. Boyd NF, Stone J, Vogt KN, Connelly BS, Martin LJ, Minkin S. Dietary fat and breast cancer risk revisited: a meta-analysis of the published literature. *Br J Cancer*. 2003;89(9):1672-85. Epub 2003/10/30.
130. Cross AJ, Leitzmann MF, Subar AF, Thompson FE, Hollenbeck AR, Schatzkin A. A prospective study of meat and fat intake in relation to small intestinal cancer. *Cancer Res*. 2008;68(22):9274-9. Epub 2008/11/18.
131. Kurahashi N, Inoue M, Iwasaki M, Sasazuki S, Tsugane AS. Dairy product, saturated fatty acid, and calcium intake and prostate cancer in a prospective cohort of Japanese men. *Cancer Epidemiol Biomarkers Prev*. 2008;17(4):930-7. Epub 2008/04/10.
132. Chow CK. *Fatty Acids in Foods and their Health Implications*. 3 ed. Boca Ranton: Taylor and Francis; 2008.
133. German JB, Dillard CJ. Saturated fats: what dietary intake? *The American journal of clinical nutrition*. 2004;80(3):550-9. Epub 2004/08/24.
134. Lichtenstein AH, Appel LJ, Brands M, Carnethon M, Daniels S, Franch HA, et al. Diet and lifestyle recommendations revision 2006: a scientific statement from the American Heart Association Nutrition Committee. *Circulation*. 2006;114(1):82-96. Epub 2006/06/21.
135. Mozaffarian D, Rimm EB, Herrington DM. Dietary fats, carbohydrate, and progression of coronary atherosclerosis in postmenopausal women. *The American journal of clinical nutrition*. 2004;80(5):1175-84. Epub 2004/11/09.
136. Scislowski V, Bauchart D, Gruffat D, Laplaud PM, Durand D. Effect of dietary n-6 and n-3 polyunsaturated fatty acids on peroxidizability of lipoproteins in steers. *Lipids*. 2005;40(12):1245-56. Epub 2006/02/16.
137. Sanchez-Bayle M, Gonzalez-Requejo A, Pelaez MJ, Morales MT, Asensio-Anton J, Anton-Pacheco E. A cross-sectional study of dietary habits and lipid profiles. The Rivas-Vaciamadrid study. *Eur J Pediatr*. 2008;167(2):149-54. Epub 2007/03/03.
138. Lovejoy JC. The influence of dietary fat on insulin resistance. *Curr Diab Rep*. 2002;2(5):435-40. Epub 2003/03/20.
139. Vessby B, Uusitupa M, Hermansen K, Riccardi G, Rivellese AA, Tapsell LC, et al. Substituting dietary saturated for monounsaturated fat impairs insulin sensitivity in healthy men and women: The KANWU Study. *Diabetologia*. 2001;44(3):312-9. Epub 2001/04/25.
140. Storlien LH, Baur LA, Kriketos AD, Pan DA, Cooney GJ, Jenkins AB, et al. Dietary fats and insulin action. *Diabetologia*. 1996;39(6):621-31. Epub 1996/06/01.
141. Pala V, Krogh V, Muti P, Chajes V, Riboli E, Micheli A, et al. Erythrocyte membrane fatty acids and subsequent breast cancer: a prospective Italian study. *J Natl Cancer Inst*. 2001;93(14):1088-95. Epub 2001/07/19.
142. Food and Nutrition Board IoMotNA. *Dietary Fats: Total Fat and Fatty Acids. Dietary Reference Intakes for Energy, Carbohydrate, Fiber, Fat, Fatty Acids, Cholesterol, Protein, and Amino Acids (Macronutrients)2005*. p. 422-541.
143. Agriculture USDoHaHSaUSDo. *Fats. Dietary Guidelines for Americans*, . Washington, DC: U.S. Government Printing Office; 2005.
144. Donald Voet JGV. *Lipid Metabolism*. In: Donald Voet JGV, editor. *Biochemistry*. 3 ed. Hoboken: John Wiley & Sons; 2004. p. 932.
145. Center C. Prostanoids and related compounds. 2011 [cited 2011 04/10/2011]; Available from: <http://www.cyberlipid.org/prost1/pros0001.htm>.
146. Hunter JE. Dietary trans fatty acids: review of recent human studies and food industry responses. *Lipids*. 2006;41(11):967-92. Epub 2007/02/01.
147. Interface Science - Reactions on Surfaces [updated 23/02/2005; cited 2011 11/10/2011]; Available from: <http://www.if.tugraz.at/Surface/Reactions%20on%20Surfaces/ReaSur1.html>.
148. Eller FJ, List GR, Teel JA, Steidley KR, Adlof RO. Preparation of spread oils meeting U.S. Food and Drug Administration Labeling requirements for trans fatty acids via pressure-controlled hydrogenation. *J Agric Food Chem*. 2005;53(15):5982-4. Epub 2005/07/21.

149. Mozaffarian D, Katan MB, Ascherio A, Stampfer MJ, Willett WC. Trans fatty acids and cardiovascular disease. *The New England journal of medicine*. 2006;354(15):1601-13. Epub 2006/04/14.
150. Kavanagh K, Jones KL, Sawyer J, Kelley K, Carr JJ, Wagner JD, et al. Trans fat diet induces abdominal obesity and changes in insulin sensitivity in monkeys. *Obesity* (Silver Spring, Md. 2007;15(7):1675-84. Epub 2007/07/20.
151. Hu FB, van Dam RM, Liu S. Diet and risk of Type II diabetes: the role of types of fat and carbohydrate. *Diabetologia*. 2001;44(7):805-17. Epub 2001/08/18.
152. van Dam RM, Willett WC, Rimm EB, Stampfer MJ, Hu FB. Dietary fat and meat intake in relation to risk of type 2 diabetes in men. *Diabetes care*. 2002;25(3):417-24. Epub 2002/03/05.
153. Chavarro JE, Stampfer MJ, Campos H, Kurth T, Willett WC, Ma J. A prospective study of trans-fatty acid levels in blood and risk of prostate cancer. *Cancer Epidemiol Biomarkers Prev*. 2008;17(1):95-101. Epub 2008/01/18.
154. Chajes V, Thiebaut AC, Rotival M, Gauthier E, Maillard V, Boutron-Ruault MC, et al. Association between serum trans-monounsaturated fatty acids and breast cancer risk in the E3N-EPIC Study. *Am J Epidemiol*. 2008;167(11):1312-20. Epub 2008/04/09.
155. Brasky TM, Till C, White E, Neuhaus ML, Song X, Goodman P, et al. Serum phospholipid fatty acids and prostate cancer risk: results from the prostate cancer prevention trial. *Am J Epidemiol*. 2011;173(12):1429-39. Epub 2011/04/27.
156. Sanchez-Villegas A, Verberne L, De Irala J, Ruiz-Canela M, Toledo E, Serra-Majem L, et al. Dietary fat intake and the risk of depression: the SUN Project. *PLoS One*. 2011;6(1):e16268. Epub 2011/02/08.
157. Oh K, Hu FB, Manson JE, Stampfer MJ, Willett WC. Dietary fat intake and risk of coronary heart disease in women: 20 years of follow-up of the nurses' health study. *Am J Epidemiol*. 2005;161(7):672-9. Epub 2005/03/23.
158. Mahfouz M. Effect of dietary trans fatty acids on the delta 5, delta 6 and delta 9 desaturases of rat liver microsomes in vivo. *Acta Biol Med Ger*. 1981;40(12):1699-705. Epub 1981/01/01.
159. Jacome-Sosa MM, Lu J, Wang Y, Ruth MR, Wright DC, Reaney MJ, et al. Increased hypolipidemic benefits of cis-9, trans-11 conjugated linoleic acid in combination with trans-11 vaccenic acid in a rodent model of the metabolic syndrome, the JCR:LA-cp rat. *Nutr Metab (Lond)*. 2010;7:60. Epub 2010/07/17.
160. FDA USFDA-. FDA food labeling: trans fatty acids in nutrition labeling; consumer research to consider nutrient content and health claims and possible footnote or disclosure statements. 2003 [updated 22/05/2009; cited 2011 04/10/2011]; *Federal Register* - 68 FR 41507]. Available from: <http://www.fda.gov/food/labelingnutrition/labelclaims/nutrientcontentclaims/ucm110202.htm>.
161. Force TFT. TRANSforming the food supply. Health Canada; 2006 [cited 2011 04/10/2011]; Available from: http://www.hc-sc.gc.ca/fn-an/nutrition/gras-trans-fats/tf-ge/tf-gt_rep-rap-eng.php.
162. George O Burr MMB, Elmer S Miller. On the fatty acids essential in nutrition. 1932;97:1-9.
163. Gerster H. Can adults adequately convert alpha-linolenic acid (18:3n-3) to eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3)? *Int J Vitam Nutr Res*. 1998;68(3):159-73. Epub 1998/06/25.
164. Bergstroem S, Danielsson H, Klenberg D, Samuelsson B. The Enzymatic Conversion of Essential Fatty Acids into Prostaglandins. *The Journal of biological chemistry*. 1964;239:PC4006-8. Epub 1964/11/01.
165. Burdge GC, Calder PC. Conversion of alpha-linolenic acid to longer-chain polyunsaturated fatty acids in human adults. *Reprod Nutr Dev*. 2005;45(5):581-97. Epub 2005/09/29.
166. Shearer GC, Harris WS, Pedersen TL, Newman JW. Detection of omega-3 oxylipins in human plasma and response to treatment with omega-3 acid ethyl esters. *Journal of lipid research*. 2010;51(8):2074-81. Epub 2009/08/13.
167. Ingenbleek Y, Jung L, Ferard G. Brassiodol, a new iodised oil for goitrous patients. *Coll Antropol*. 1998;22(1):51-62. Epub 1999/03/31.
168. Damsgaard CT, Lauritzen L, Kjaer TM, Holm PM, Fruekilde MB, Michaelsen KF, et al. Fish oil supplementation modulates immune function in healthy infants. *J Nutr*. 2007;137(4):1031-6. Epub 2007/03/22.
169. Evans AR, Junger H, Southall MD, Nicol GD, Sorkin LS, Broome JT, et al. Isoprostanes, novel eicosanoids that produce nociception and sensitize rat sensory neurons. *J Pharmacol Exp Ther*. 2000;293(3):912-20. Epub 2000/06/28.
170. Brenda Davis PK-E, editor. Achieving Optimal Essential Fatty Acid Status in Vegetarians: Current Knowledge and Practical Implications. 4th International Congress of Vegetarian Nutrition; 2002 08-11/04/2002; Loma.
171. Trivedi B. The good, the bad, and the unhealthy. *New Scientist*. 2006(2570):42-9.
172. von Schacky C. The role of omega-3 fatty acids in cardiovascular disease. *Curr Atheroscler Rep*. 2003;5(2):139-45. Epub 2003/02/08.

173. Wang C, Harris WS, Chung M, Lichtenstein AH, Balk EM, Kupelnick B, et al. n-3 Fatty acids from fish or fish-oil supplements, but not alpha-linolenic acid, benefit cardiovascular disease outcomes in primary- and secondary-prevention studies: a systematic review. *The American journal of clinical nutrition*. 2006;84(1):5-17. Epub 2006/07/11.
174. Hulbert AJ. Life, death and membrane bilayers. *J Exp Biol*. 2003;206(Pt 14):2303-11. Epub 2003/06/11.
175. Harris WS. n-3 fatty acids and serum lipoproteins: human studies. *The American journal of clinical nutrition*. 1997;65(5 Suppl):1645S-54S. Epub 1997/05/01.
176. Morris MC, Sacks F, Rosner B. Does fish oil lower blood pressure? A meta-analysis of controlled trials. *Circulation*. 1993;88(2):523-33. Epub 1993/08/01.
177. Davidson MH, Stein EA, Bays HE, Maki KC, Doyle RT, Shalwitz RA, et al. Efficacy and tolerability of adding prescription omega-3 fatty acids 4 g/d to simvastatin 40 mg/d in hypertriglyceridemic patients: an 8-week, randomized, double-blind, placebo-controlled study. *Clin Ther*. 2007;29(7):1354-67. Epub 2007/09/11.
178. Roche HM, Gibney MJ. Postprandial triacylglycerolaemia: the effect of low-fat dietary treatment with and without fish oil supplementation. *Eur J Clin Nutr*. 1996;50(9):617-24. Epub 1996/09/01.
179. Pignier C, Revenaz C, Raully-Lestienne I, Cussac D, Delhon A, Gardette J, et al. Direct protective effects of poly-unsaturated fatty acids, DHA and EPA, against activation of cardiac late sodium current: a mechanism for ischemia selectivity. *Basic Res Cardiol*. 2007;102(6):553-64. Epub 2007/09/25.
180. Wang B, Trayhurn P. Acute and prolonged effects of TNF-alpha on the expression and secretion of inflammation-related adipokines by human adipocytes differentiated in culture. *Pflugers Arch*. 2006;452(4):418-27.
181. Murnaghan MF. Effect of fatty acids on the ventricular arrhythmia threshold in the isolated heart of the rabbit. *Br J Pharmacol*. 1981;73(4):909-15. Epub 1981/08/01.
182. Mita T, Watada H, Ogihara T, Nomiyama T, Ogawa O, Kinoshita J, et al. Eicosapentaenoic acid reduces the progression of carotid intima-media thickness in patients with type 2 diabetes. *Atherosclerosis*. 2007;191(1):162-7. Epub 2006/04/18.
183. McKenney JM, Sica D. Prescription omega-3 fatty acids for the treatment of hypertriglyceridemia. *Am J Health Syst Pharm*. 2007;64(6):595-605. Epub 2007/03/14.
184. Garrido-Sanchez L, Garcia-Fuentes E, Rojo-Martinez G, Cardona F, Soriguer F, Tinahones FJ. Inverse relation between levels of anti-oxidized-LDL antibodies and eicosapentanoic acid (EPA). *The British journal of nutrition*. 2008;100(3):585-9. Epub 2008/02/07.
185. Wall R, Ross RP, Fitzgerald GF, Stanton C. Fatty acids from fish: the anti-inflammatory potential of long-chain omega-3 fatty acids. *Nutr Rev*. 2010;68(5):280-9. Epub 2010/05/27.
186. Fortin PR, Lew RA, Liang MH, Wright EA, Beckett LA, Chalmers TC, et al. Validation of a meta-analysis: the effects of fish oil in rheumatoid arthritis. *J Clin Epidemiol*. 1995;48(11):1379-90. Epub 1995/11/01.
187. Ruggiero C, Lattanzio F, Lauretani F, Gasperini B, Andres-Lacueva C, Cherubini A. Omega-3 polyunsaturated fatty acids and immune-mediated diseases: inflammatory bowel disease and rheumatoid arthritis. *Current pharmaceutical design*. 2009;15(36):4135-48. Epub 2010/01/01.
188. Naliwaiko K, Araujo RL, da Fonseca RV, Castilho JC, Andreatini R, Bellissimo MI, et al. Effects of fish oil on the central nervous system: a new potential antidepressant? *Nutr Neurosci*. 2004;7(2):91-9. Epub 2004/07/29.
189. Nemets H, Nemets B, Apter A, Bracha Z, Belmaker RH. Omega-3 treatment of childhood depression: a controlled, double-blind pilot study. *Am J Psychiatry*. 2006;163(6):1098-100. Epub 2006/06/03.
190. Green P, Hermesh H, Monselise A, Marom S, Presburger G, Weizman A. Red cell membrane omega-3 fatty acids are decreased in nondepressed patients with social anxiety disorder. *Eur Neuropsychopharmacol*. 2006;16(2):107-13. Epub 2005/10/26.
191. Samuelsson B. Leukotrienes: mediators of immediate hypersensitivity reactions and inflammation. *Science (New York, NY)*. 1983;220(4597):568-75. Epub 1983/05/06.
192. Mischoulon D, Papakostas GI, Dording CM, Farabaugh AH, Sonawalla SB, Agoston AM, et al. A double-blind, randomized controlled trial of ethyl-eicosapentaenoate for major depressive disorder. *J Clin Psychiatry*. 2009;70(12):1636-44. Epub 2009/08/28.
193. Carney RM, Freedland KE, Rubin EH, Rich MW, Steinmeyer BC, Harris WS. Omega-3 augmentation of sertraline in treatment of depression in patients with coronary heart disease: a randomized controlled trial. *JAMA*. 2009;302(15):1651-7. Epub 2009/10/22.
194. Huan M, Hamazaki K, Sun Y, Itomura M, Liu H, Kang W, et al. Suicide attempt and n-3 fatty acid levels in red blood cells: a case control study in China. *Biol Psychiatry*. 2004;56(7):490-6. Epub 2004/09/29.

195. Amminger GP, Schafer MR, Papageorgiou K, Klier CM, Cotton SM, Harrigan SM, et al. Long-chain omega-3 fatty acids for indicated prevention of psychotic disorders: a randomized, placebo-controlled trial. *Arch Gen Psychiatry*. 2010;67(2):146-54. Epub 2010/02/04.
196. Puri BK. High-resolution magnetic resonance imaging sinc-interpolation-based subvoxel registration and semi-automated quantitative lateral ventricular morphology employing threshold computation and binary image creation in the study of fatty acid interventions in schizophrenia, depression, chronic fatigue syndrome and Huntington's disease. *Int Rev Psychiatry*. 2006;18(2):149-54. Epub 2006/06/17.
197. Johnson M, Ostlund S, Fransson G, Kadesjo B, Gillberg C. Omega-3/omega-6 fatty acids for attention deficit hyperactivity disorder: a randomized placebo-controlled trial in children and adolescents. *J Atten Disord*. 2009;12(5):394-401. Epub 2008/05/02.
198. Augustsson K, Michaud DS, Rimm EB, Leitzmann MF, Stampfer MJ, Willett WC, et al. A prospective study of intake of fish and marine fatty acids and prostate cancer. *Cancer Epidemiol Biomarkers Prev*. 2003;12(1):64-7. Epub 2003/01/24.
199. de Deckere EA. Possible beneficial effect of fish and fish n-3 polyunsaturated fatty acids in breast and colorectal cancer. *Eur J Cancer Prev*. 1999;8(3):213-21. Epub 1999/08/12.
200. MacLean CH, Newberry SJ, Mojica WA, Khanna P, Issa AM, Sutorp MJ, et al. Effects of omega-3 fatty acids on cancer risk: a systematic review. *JAMA*. 2006;295(4):403-15. Epub 2006/01/26.
201. Smith KL, Guentzel JL. Mercury concentrations and omega-3 fatty acids in fish and shrimp: Preferential consumption for maximum health benefits. *Mar Pollut Bull*. 2010;60(9):1615-8. Epub 2010/07/17.
202. Ulven SM, Kirkhus B, Lamglait A, Basu S, Elind E, Haider T, et al. Metabolic effects of krill oil are essentially similar to those of fish oil but at lower dose of EPA and DHA, in healthy volunteers. *Lipids*. 2011;46(1):37-46. Epub 2010/11/03.
203. Erasmus U. *Fats and Oils*. Vancouver: Alive books; 1986.
204. NIH TPFASIG-. *Essential Fats in food oils 2011* [updated 01/01/2010; cited 2011 04/10/2011]; Available from: <http://efaeducation.nih.gov/sig/esstable.html>.
205. CSPI CfSitPI-. *Egg Producers Deceive Consumers, Violate Law with Bogus Omega-3 Claims*. 2007 [updated 21/06/2007; cited 2011 04/10/2011]; Available from: <http://www.cspinet.org/new/200706211.html>.
206. Daly J. *Egg Taste Test Says Homegrown And Factory Eggs Taste The Same*. The Huffington Post; 2010 [updated 08/03/2010; cited 2011 04/10/2011]; Available from: http://www.huffingtonpost.com/2010/06/03/egg-taste-test-says-no-di_n_599286.html.
207. Duckett SK, Neel JP, Fontenot JP, Clapham WM. Effects of winter stocker growth rate and finishing system on: III. Tissue proximate, fatty acid, vitamin, and cholesterol content. *J Anim Sci*. 2009;87(9):2961-70. Epub 2009/06/09.
208. FDA USFaDA-. *FDA Announces Qualified Health Claims for Omega-3 Fatty Acids*. 2004 [updated 18/06/2009; cited 2011 04/10/2011]; Press Release P04-89]. Available from: <http://www.fda.gov/SiteIndex/ucm108351.htm>.
209. FDA USFaDA-. *Overview of Scientific Information on the Effect of Fish Consumption and Omega-3 Fatty Acids on Cardiovascular Disease*. 2009 [updated 06/18/2011; cited 2011 05/10/2011]; Available from: <http://www.fda.gov/Food/FoodSafety/Product-SpecificInformation/Seafood/FoodbornePathogensContaminants/Methylmercury/ucm153053.htm>.
210. Willett WC. The role of dietary n-6 fatty acids in the prevention of cardiovascular disease. *J Cardiovasc Med (Hagerstown)*. 2007;8 Suppl 1:S42-5. Epub 2007/10/19.
211. Calder PC. n-3 polyunsaturated fatty acids, inflammation, and inflammatory diseases. *The American journal of clinical nutrition*. 2006;83(6 Suppl):1505S-19S. Epub 2006/07/18.
212. Calder PC. Session 3: Joint Nutrition Society and Irish Nutrition and Dietetic Institute Symposium on 'Nutrition and autoimmune disease' PUFA, inflammatory processes and rheumatoid arthritis. *The Proceedings of the Nutrition Society*. 2008;67(4):409-18. Epub 2008/10/14.
213. Lands WE. Dietary fat and health: the evidence and the politics of prevention: careful use of dietary fats can improve life and prevent disease. *Ann N Y Acad Sci*. 2005;1055:179-92. Epub 2006/01/03.
214. Simopoulos AP. The importance of the ratio of omega-6/omega-3 essential fatty acids. *Biomed Pharmacother*. 2002;56(8):365-79. Epub 2002/11/22.
215. Simopoulos AP. Importance of the ratio of omega-6/omega-3 essential fatty acids: evolutionary aspects. *World Rev Nutr Diet*. 2003;92:1-22. Epub 2003/10/29.

216. Sonestedt E, Ericson U, Gullberg B, Skog K, Olsson H, Wirfalt E. Do both heterocyclic amines and omega-6 polyunsaturated fatty acids contribute to the incidence of breast cancer in postmenopausal women of the Malmo diet and cancer cohort? *Int J Cancer*. 2008;123(7):1637-43. Epub 2008/07/19.
217. Berquin IM, Min Y, Wu R, Wu J, Perry D, Cline JM, et al. Modulation of prostate cancer genetic risk by omega-3 and omega-6 fatty acids. *The Journal of clinical investigation*. 2007;117(7):1866-75. Epub 2007/07/04.
218. Cleland LG, James MJ, Proudman SM. Fish oil: what the prescriber needs to know. *Arthritis Res Ther*. 2006;8(1):202. Epub 2006/03/18.
219. Lee HJ, Rao JS, Rapoport SI, Bazinet RP. Antimanic therapies target brain arachidonic acid signaling: lessons learned about the regulation of brain fatty acid metabolism. *Prostaglandins Leukot Essent Fatty Acids*. 2007;77(5-6):239-46. Epub 2007/11/29.
220. Broughton KS, Johnson CS, Pace BK, Liebman M, Kleppinger KM. Reduced asthma symptoms with n-3 fatty acid ingestion are related to 5-series leukotriene production. *The American journal of clinical nutrition*. 1997;65(4):1011-7. Epub 1997/04/01.
221. Gunstone C. Qof. *Br J Gen Pract*. 2007;57(542):748. Epub 2007/09/01.
222. Bjursell M, Ahnmark A, Bohlooly YM, William-Olsson L, Rhedin M, Peng XR, et al. Opposing effects of adiponectin receptors 1 and 2 on energy metabolism. *Diabetes*. 2007;56(3):583-93.
223. Cunnane SC. Problems with essential fatty acids: time for a new paradigm? *Prog Lipid Res*. 2003;42(6):544-68. Epub 2003/10/16.
224. Phinney SD, Odin RS, Johnson SB, Holman RT. Reduced arachidonate in serum phospholipids and cholesteryl esters associated with vegetarian diets in humans. *The American journal of clinical nutrition*. 1990;51(3):385-92. Epub 1990/03/01.
225. Technologies L. Mead acid. [updated 05/08/2011; cited 2011 04/10/2011]; Available from: http://www.lipomics.com/fatty_acids/5-8-11-eicosatrienoic-acid.
226. Griffin BA. How relevant is the ratio of dietary n-6 to n-3 polyunsaturated fatty acids to cardiovascular disease risk? Evidence from the OPTILIP study. *Curr Opin Lipidol*. 2008;19(1):57-62. Epub 2008/01/17.
227. Simopoulos AP, Leaf A, Salem N, Jr. Workshop statement on the essentiality of and recommended dietary intakes for Omega-6 and Omega-3 fatty acids. *Prostaglandins Leukot Essent Fatty Acids*. 2000;63(3):119-21. Epub 2000/09/19.
228. Hibbeln JR, Nieminen LR, Blasbalg TL, Riggs JA, Lands WE. Healthy intakes of n-3 and n-6 fatty acids: estimations considering worldwide diversity. *The American journal of clinical nutrition*. 2006;83(6 Suppl):1483S-93S. Epub 2006/07/18.
229. Simopoulos AP. Evolutionary aspects of diet and essential fatty acids. *World Rev Nutr Diet*. 2001;88:18-27. Epub 2002/04/09.
230. Hooper L, Thompson RL, Harrison RA, Summerbell CD, Ness AR, Moore HJ, et al. Risks and benefits of omega 3 fats for mortality, cardiovascular disease, and cancer: systematic review. *BMJ*. 2006;332(7544):752-60. Epub 2006/03/28.
231. Serhan CN. Novel eicosanoid and docosanoid mediators: resolvins, docosatrienes, and neuroprotectins. *Current opinion in clinical nutrition and metabolic care*. 2005;8(2):115-21. Epub 2005/02/18.
232. Christie WW. Eicosanoids and related compounds - an introduction. [updated 01/03/2011; cited 2011 11/10/2011]; Available from: <http://lipidlibrary.aocs.org/lipids/eicintro/index.htm>.
233. Eicosanoids. [updated 01/03/2011; cited 2011 11/10/2011]; Available from: http://www.gremi.asso.fr/mediator_inflammation-uk.html
234. Lands WE. Biochemistry and physiology of n-3 fatty acids. *FASEB J*. 1992;6(8):2530-6. Epub 1992/05/01.
235. Tilley SL, Coffman TM, Koller BH. Mixed messages: modulation of inflammation and immune responses by prostaglandins and thromboxanes. *The Journal of clinical investigation*. 2001;108(1):15-23. Epub 2001/07/04.
236. Calder PC. n-3 fatty acids, inflammation, and immunity--relevance to postsurgical and critically ill patients. *Lipids*. 2004;39(12):1147-61. Epub 2005/03/02.
237. Novgorodtseva TP, Karaman YK, Zhukova NV, Lobanova EG, Antonyuk MV, Kantur TA. Composition of fatty acids in plasma and erythrocytes and eicosanoids level in patients with metabolic syndrome. *Lipids Health Dis*. 2011;10:82. Epub 2011/05/21.
238. Piomelli D. Arachidonic Acid. 2000 [cited 2011 04/10/2011]; Available from: <http://www.acnp.org/g4/GN401000059/Default.htm>.
239. Fritsche K. Fatty acids as modulators of the immune response. *Annu Rev Nutr*. 2006;26:45-73. Epub 2006/07/20.

240. Su KP, Huang SY, Chiu CC, Shen WW. Omega-3 fatty acids in major depressive disorder. A preliminary double-blind, placebo-controlled trial. *Eur Neuropsychopharmacol.* 2003;13(4):267-71. Epub 2003/07/31.
241. Johnson MM, Swan DD, Surette ME, Stegner J, Chilton T, Fonteh AN, et al. Dietary supplementation with gamma-linolenic acid alters fatty acid content and eicosanoid production in healthy humans. *J Nutr.* 1997;127(8):1435-44. Epub 1997/08/01.
242. Pace-Asciak CR, Hahn S, Diamandis EP, Soleas G, Goldberg DM. The red wine phenolics trans-resveratrol and quercetin block human platelet aggregation and eicosanoid synthesis: implications for protection against coronary heart disease. *Clinica chimica acta; international journal of clinical chemistry.* 1995;235(2):207-19. Epub 1995/03/31.
243. Bazan NG. Omega-3 fatty acids, pro-inflammatory signaling and neuroprotection. *Current opinion in clinical nutrition and metabolic care.* 2007;10(2):136-41. Epub 2007/02/08.
244. Serhan CN, Gotlinger K, Hong S, Arita M. Resolvins, docosatrienes, and neuroprotectins, novel omega-3-derived mediators, and their aspirin-triggered endogenous epimers: an overview of their protective roles in catabasis. *Prostaglandins Other Lipid Mediat.* 2004;73(3-4):155-72. Epub 2004/08/05.
245. Brites P, Waterham HR, Wanders RJ. Functions and biosynthesis of plasmalogens in health and disease. *Biochimica et biophysica acta.* 2004;1636(2-3):219-31. Epub 2004/05/29.
246. Wallner S, Schmitz G. Plasmalogens the neglected regulatory and scavenging lipid species. *Chemistry and physics of lipids.* 2011;164(6):573-89. Epub 2011/07/05.
247. Labadaridis I, Moraitou M, Theodoraki M, Triantafyllidis G, Sarafidou J, Michelakakis H. Plasmalogen levels in full-term neonates. *Acta Paediatr.* 2009;98(4):640-2. Epub 2009/03/18.
248. Moser AB, Steinberg SJ, Watkins PA, Moser HW, Ramaswamy K, Siegmund KD, et al. Human and great ape red blood cells differ in plasmalogen levels and composition. *Lipids Health Dis.* 2011;10:101. Epub 2011/06/18.
249. Acar N, Berdeaux O, Juaneda P, Gregoire S, Cabaret S, Joffre C, et al. Red blood cell plasmalogens and docosahexaenoic acid are independently reduced in primary open-angle glaucoma. *Experimental eye research.* 2009;89(6):840-53. Epub 2009/07/25.
250. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia.* 1985;28(7):412-9.
251. Dodge JT, Mitchell C, Hanahan DJ. The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes. *Archives of biochemistry and biophysics.* 1963;100:119-30.
252. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry.* 1976;72:248-54.
253. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 1970;227(5259):680-5.
254. Mihara M, Uchiyama M. [Evaluation of thiobarbituric acid (TBA) value as an index of lipid peroxidation in CCl4-intoxicated rat liver (author's transl)]. *Yakugaku Zasshi.* 1981;101(3):221-6. Epub 1981/03/01.
255. Levine RL, Garland D, Oliver CN, Amici A, Climent I, Lenz AG, et al. Determination of carbonyl content in oxidatively modified proteins. *Methods Enzymol.* 1990;186:464-78. Epub 1990/01/01.
256. Folch J, Lees M. Proteolipides, a new type of tissue lipoproteins; their isolation from brain. *The Journal of biological chemistry.* 1951;191(2):807-17. Epub 1951/08/01.
257. Nofer JR, Kehrel B, Fobker M, Levkau B, Assmann G, von Eckardstein A. HDL and arteriosclerosis: beyond reverse cholesterol transport. *Atherosclerosis.* 2002;161(1):1-16.
258. Packard C, Caslake M, Shepherd J. The role of small, dense low density lipoprotein (LDL): a new look. *International journal of cardiology.* 2000;74 Suppl 1:S17-22.
259. Saito M, Ishimitsu T, Minami J, Ono H, Ohru M, Matsuoka H. Relations of plasma high-sensitivity C-reactive protein to traditional cardiovascular risk factors. *Atherosclerosis.* 2003;167(1):73-9.
260. Labarrere CA, Zaloga GP. C-reactive protein: from innocent bystander to pivotal mediator of atherosclerosis. *The American journal of medicine.* 2004;117(7):499-507.
261. Bo S, Gambino R, Pagani A, Guidi S, Gentile L, Cassader M, et al. Relationships between human serum resistin, inflammatory markers and insulin resistance. *International journal of obesity (2005).* 2005;29(11):1315-20.
262. Bastard JP, Maachi M, Lagathu C, Kim MJ, Caron M, Vidal H, et al. Recent advances in the relationship between obesity, inflammation, and insulin resistance. *European cytokine network.* 2006;17(1):4-12.
263. Winer JC, Zern TL, Taksali SE, Dziura J, Cali AM, Wollschlager M, et al. Adiponectin in childhood and adolescent obesity and its association with inflammatory markers and components of the metabolic syndrome. *The Journal of clinical endocrinology and metabolism.* 2006;91(11):4415-23.

- 264. Miyazawa-Hoshimoto S, Takahashi K, Bujo H, Hashimoto N, Saito Y. Elevated serum vascular endothelial growth factor is associated with visceral fat accumulation in human obese subjects. *Diabetologia*. 2003;46(11):1483-8.
- 265. Hopfl G, Ogunshola O, Gassmann M. HIFs and tumors--causes and consequences. *Am J Physiol Regul Integr Comp Physiol*. 2004;286(4):R608-23.
- 266. Silha JV, Krsek M, Sucharda P, Murphy LJ. Angiogenic factors are elevated in overweight and obese individuals. *International journal of obesity (2005)*. 2005;29(11):1308-14.
- 267. Assies J, Pouwer F, Lok A, Mocking RJ, Bockting CL, Visser I, et al. Plasma and erythrocyte fatty acid patterns in patients with recurrent depression: a matched case-control study. *PLoS One*. 2010;5(5):e10635. Epub 2010/05/26.
- 268. Shannon J, O'Malley J, Mori M, Garzotto M, Palma AJ, King IB. Erythrocyte fatty acids and prostate cancer risk: a comparison of methods. *Prostaglandins Leukot Essent Fatty Acids*. 2010;83(3):161-9. Epub 2010/07/16.
- 269. Mertens A, Holvoet P. Oxidized LDL and HDL: antagonists in atherothrombosis. *Faseb J*. 2001;15(12):2073-84.
- 270. Reinehr T, Andler W. Changes in the atherogenic risk factor profile according to degree of weight loss. *Archives of disease in childhood*. 2004;89(5):419-22.
- 271. de Luca C, Olefsky JM. Inflammation and insulin resistance. *FEBS letters*. 2008;582(1):97-105.

