Universidade Federal do Rio Grande do Sul (UFRGS)

Instituto de Ciências Básicas da Saúde (ICBS)

Programa de Pós-Graduação em Ciências Biológicas: Bioquímica

Tese de Doutorado

USO DE FERRAMENTAS DE BIOINFORMÁTICA NA ANÁLISE DA EXPRESSÃO DE GENES ANTIOXIDANTES E DE GENES DOS FENÓTIPOS M1 E M2 EM ATEROSCLEROSE

Porto Alegre – RS – Brasil

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Para os crentes, Deus está no princípio das coisas. Para os cientistas, no final de toda reflexão.

Max Karl Ernest Ludwig Planck *(in memorian)*, 1858 - 1947

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Tese dedicada ao grande Prof. Perry, um exímio conhecedor de Bioquímica e uma referência na área. Um exemplo a ser seguido.

Dedicado a Marcos Luiz Santos Perry *(in memorian)*, 1948 - 2010

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ÍNDICE

PARTE I

RESUMO

A aterosclerose é uma doença pró-inflamatória, caracterizada por disfunção endotelial e pela presença de placa de ateroma, formada pela fagocitose de oxLDL por macrófagos da região subíntima. As espécies reativas apresentam um papel importante na doença, sendo responsáveis diretos pela oxidação da LDL. Os macrófagos podem apresentar dois fenótipos, o classicamente ativado, M1 (pró-inflamatório), e o alternativamente ativado, M2 (anti-inflamatório). Entretanto, o papel desses fenótipos na aterosclerose ainda carece de um maior entendimento. Portanto, nosso objetivo foi, em um primeiro momento, revisar os dados presentes na literatura sobre oxidação de LDL e fenótipos de macrófagos em aterosclerose. Em um segundo momento, objetivamos comparar (através de estudo de bioinformática) as expressões de grupos de genes antioxidantes (HAG), de genes relacionados ao fenótipo M1 e de genes relacionados ao fenótipo M2 entre macrófagos de pessoas com aterosclerose e de pessoas saudáveis e entre placas de aterosclerose humanas em estágio avançado e em estágio inicial. Os dados de expressão foram obtidos do repositório GEO (http://www.ncbi.nlm.nih.gov/geo/), enquanto as interações funcionais foram obtidas com os programas STRING (http://string-db.org/) e Medusa (http://coot.embl.de/medusa/). As análises estatísticas foram conduzidas com o programa ViaComplex (http://lief.if.ufrgs.br/pub/biosoftwares/viacomplex/) ze com a plataforma GSEA (http://www.broadinstitute.org/gsea/index.jsp). A expressão dos grupos de genes HAG e M1 aumentou nas placas em estágio avançado em comparação às placas em estágio inicial. A expressão dos grupos de genes HAG, M1 e M2 aumentou nos macrófagos de pessoas com aterosclerose em comparação com os macrófagos de pessoas saudáveis, mas somente o grupo de genes M1 teve sua expressão aumentada em células espumosas (*foam cells*) de pessoas com aterosclerose em comparação com pessoas saudáveis. Por outro lado, houve uma diminuição na expressão do grupo de genes M1 em *foam cells* de pessoas saudáveis em comparação com macrófagos do mesmo grupo de indivíduos. Portanto, nossos resultados sugerem que, diferente do que acontece em câncer, na aterosclerose não há uma polarização dos fenótipos de macrófagos. Na verdade, ambos estão aumentados e mais estudos são necessários para melhor elucidar os mecanismos envolvidos. Palavras-chave: antioxidantes, aterosclerose, macrófagos, polarização, M1/M2.

ABSTRACT

Atherosclerosis is a pro-inflammatory disease, which is characterized by endothelial dysfunction and atheroma plaque formation, as a result of oxLDL phagocytosis by macrophages in subintima region. Reactive species play an important role, being involved with the LDL oxidation process. Macrophages can present two phenotypes, classically activated, M1 (pro-inflammatory), and the alternatively activated, M2 (anti-inflammatory). However, the role of these phenotypes needs to be better explained. Therefore, our objective is to review the literature data about LDL oxidation and macrophage phenotypes in atherosclerosis. Thereafter, we aimed to compare, through bioinformatics study, the expression of human antioxidant genes (HAG), M1 phenotype-related genes and M2 phenotype-related genes groups between healthy people macrophages and atherosclerotic people macrophages, and between human advanced atherosclerotic plaques and human initial atherosclerotic plaques. Expression data were obtained from GEO (http://www.ncbi.nlm.nih.gov/geo/), while functional interactions were from STRING (http://string-db.org/) and Medusa (http://coot.embl.de/medusa/). The statistical analysis was conducted with ViaComplex (http://lief.if.ufrgs.br/pub/biosoftwares/viacomplex/) and GSEA (http://www.broadinstitute.org/gsea/index.jsp). The expression of HAG e M1 groups increased in advanced plaques compared to initial plaques, while the expression of HAG, M1 e M2 groups increased in atherosclerotic people macrophages compared to healthy people macrophages. Nevertheless, only M1 group had its expression elevated in atherosclerotic people *foam cells* compared to healthy people *foam cells*. On the other hand, there was a decreased expression of M1 group in healthy people *foam cells* compared to the macrophages from the same individuals set. Thus, our results suggest that in atherosclerosis there is not a macrophage phenotype polarization, differently of what happens for cancer. Actually, both phenotypes are increased and more studies are needed to better elucidate the involved mechanisms. Key-words: antioxidants, atherosclerosis, macrophages, polarization, M1/M2.

LISTA DE ABREVIATURAS E SIGLAS

Apo B-100 – Apolipoproteína B-100

ATF-1 – Fator de transcrição de ativação 1

CAT – Catalase

CD14 – Molécula CD14

CD16 – Molécula CD16

CD18 – Molécula CD18

CD36 – Molécula CD36

EARLY – Avaliação Endotelial de Risco de Lipídios em Jovens

EDRF – Fator de relaxamento derivado do endotélio

eNOS – Óxido nítrico sintase endotelial (constituinte)

GliSODin – Superóxido dismutase vegetal associada com gliadina

GLRX - Glutaredoxina

GMPc – Guanosina monofostato cíclica

GSH – Glutationa (forma reduzida da glutationa)

GSSG – Dissulfeto de glutationa (forma oxidada da glutationa)

GPx – Glutationa peroxidase

HAG – Genes antioxidantes humanos

hsCRP – Proteína reativa C de alta sensibilidade

IFN-γ – Interferon gama

IL-1 – Interleucina 1

IL-1β – Interleucina 1β

IL-4 – Interleucina 4

IL-6 – Interleucina 6

IL-10 – Interleucina 10

IL-12 – Interleucina 12

IL-13 – Interleucina 13

iNOS – Óxido nítrico sintase induzível

IUBMB – União Internacional de Bioquímica e Biologia Molecular

IUPAC – União Internacional de Química Pura e Aplicada

LDL – Lipoproteína de baixa densidade

LPS – Lipopolissacarídeo bacteriano

M0 – Macrófagos não ativados

M1 – Macrófagos de fenótipo1 (pró-inflamatório)

M2 – Macrófagos de fenótipo 2 (anti-inflamatório)

Mox - Macrófagos

MMP – Metaloproteinase de matriz extracelular

MMP-9 – Metaloproteinase de matriz extracelular 9

MT1F – Metalotioneína 1F

MT1G – Metalotioneína 1G

MT1H – Metalotioneína 1H

MT1X – Metalotioneína 1X

MT2A –Metalotioneína 2A

NADPH – Nicotinamida adenina dinucleotídeo fosfato (forma reduzida)

nNOS – Óxido nítrico sintase neuronal (constituinte)

NOX – NADPH oxidase

NOX1 – NADPH oxidase 1

NOS – Óxido nítrico sintase

NOS1 – Óxido nítrico sintase 1

NOS3 – Óxido nítrico sintase 3

NFκB – Fator nuclear kappa B

NrF2 – Fator 2 relacionado ao fator nuclear eritróide 2

NSCLC – Câncer de pulmão de não pequenas células

oxLDL – Lipoproteína de baixa densidade oxidada

Prx – Peroxirredoxina

RNS – Espécie(s) reativa(s) de nitrogênio

ROS – Espécie(s) reativa(s) de oxigênio

RS – Espécie(s) reativa(s)

SOD – Superóxido dismutase

SOD1 – Superóxido dismutase 1

SOD2 – Superóxido dismutase 2

SR-A1 – Gene do ativador de ácido ribonucleico de receptor esteróide

TGF-β – Fator de transformação do crescimento beta

TNF-α – Fator de necrose tumoral alfa

Trx – Tiorredoxina

Trx-80 – Tiorredoxina 80

TrxR – Tiorredoxina redutase

TXNDC5 – Tioredoxina contendo domínio 5

VEAPS – Estudo de Prevenção de Aterosclerose por Vitamina E

VEGF – Fator de crescimento endotelial vascular

VSMC – Célula muscular lisa vascular

WHO – Organização Mundial da Saúde

INTRODUÇÃO

ATEROSCLEROSE

A aterosclerose é uma doença cardiovascular caracterizada pela formação de placas de ateroma, popularmente conhecidas como "placas de gordura", nas paredes dos vasos sanguíneos. No entanto, a formação dessas placas é bastante complexa, sendo difícil determinar o mecanismo exato que dá origem ao processo, mesmo que seja uma doença muito estudada e com vários mecanismos relacionados bem descritos (Stocker and Keaney, 2004).

As paredes arteriais são formadas por uma camada unicelular de endotélio (região íntima), uma camada multicelular contendo células musculares lisas (camada média) e uma camada mais externa contendo tecido conjuntivo (camada adventícia). Fisiologicamente, o endotélio tem o papel de regular o tônus vascular em resposta às variações de fluxo e pressão sanguíneos. O aumento do fluxo de sangue no vaso gera um estresse de cisalhamento (força de atrito) nas células endoteliais, fazendo com que sinais sejam disparados intracelularmente, levando à formação de uma molécula responsável pelo relaxamento do vaso. Inicialmente, quando o fenômeno foi descrito, tal molécula foi chamada de fator de relaxamento derivado do endotélio, EDRF (do inglês *endothelium derived relaxing factor*), sendo, posteriormente, descrita como sendo um radical centrado em nitrogênio, o óxido nítrico ($^{\cdot}NO$). O óxido nítrico, sintetizado na célula endotelial, difunde-se através das membranas celulares até chegar ao citosol de uma célula muscular lisa vascular, VSMC (do inglês *vascular smooth muscle cell*), onde ativa a enzima guanilato ciclase, que por sua vez catalisa a formação de guanosina monofosfato cíclico (GMPc). A GMPc interage com receptores que induzem o relaxamento da VSMC. O relaxamento das VSMC leva a uma dilatação do vaso (vasodilatação), evitando, dessa forma, aumentos incontroláveis da pressão vascular.

Moléculas como a angiotensina agem se opondo ao ·NO, induzindo a contração das VSMC, o que resulta em uma constrição dos vasos (vasoconstrição). A descrição do ·NO e dos mecanismos relacionados foi de uma importância tão grande para ciência, que rendeu o Prêmio Nobel em Fisiologia ou Medicina de 1998 aos pesquisadores Robert F. Furchgott, Louis J. Ignarro e Ferid Murad (Ignarro, 2002). A aterosclerose é uma doença altamente relacionada à disfunção endotelial, que pode ser causada por vários fatores, como o estresse oxidativo, o que resulta em um vaso não responsivo às alterações de fluxo sanguíneo e a outros estímulos (Ignarro, 2002; Stocker and Keaney, 2004).

Além da disfunção endotelial, outro fator classicamente associado à doença é a oxidação de lipoproteínas de baixa densidade (LDL). Um incremento na produção de espécies reativas leva à formação de uma LDL oxidada (oxLDL) na região subíntima (espaço entre o endotélio e as VSMC). A oxLDL pode então ser fagocitada por macrófagos da região (monócitos teciduais), os quais passam a aumentar de volume, adquirindo a forma de *foam cells* (células espumosas). Adicionalmente, a oxLDL pode se ligar a receptores de membrana presentes nos macrófagos, estimulando uma via de sinalização que ativa o fator de transcrição *kappa* B (NFκB), o qual está relacionado com a transcrição de genes pró-inflamatórios. Logo, mais monócitos são atraídos para região, são diferenciados em macrófagos, fagocitam mais oxLDL e adquirem a forma de *foam cells* , o que resulta no aumento da quantidade e do tamanho de células. Interessantemente, os macrófagos não são os únicos a reconhecerem as oxLDL, mas os VSMC também apresentam receptores capazes de tal reconhecimento. O resultado disso é que essas células musculares perdem sua característica fisiológica e passam a apresentar características de macrófagos, com ativação de NFκB e incremento na indução do processo inflamatório. Em resposta às oxLDL, as VSMC passam a sintetizar metaloproteinases de matriz extracelular, MMP (do inglês *matrix metalloproteinases*), também conhecidas como gelatinases, que agem degradando a matriz extracelular, o que

permite que as células musculares se difundam da média para a íntima. Por fim, o conjunto de todos esses eventos resulta na formação da placa de ateroma, a qual diminui a luz do vaso. Com um calibre vascular menor e com o endotélio danificado, o fluxo sanguíneo fica prejudicado e a pressão arterial aumenta. As MMP desempenham um papel importante também no rompimento da placa e liberação de um trombo, que passa a circular no organismo (trombose) e, perigosamente, pode obstruir a irrigação sanguínea em regiões com artérias de menor calibre, como a artéria coronária, responsável pelo suprimento do coração. A obstrução da coronária é conhecida como doença arterial coronariana, a qual pode levar então ao infarto agudo do miocárdio. Portanto, pode-se dizer, em síntese, que a patogenia da aterosclerose está relacionada ao estresse oxidativo e aos processos pró-inflamatórios (Chen et al., 2013; Kim et al., 2011; Kleinegris et al., 2012; Stocker and Keaney, 2004; Yang et al., 2012).

Figura 1: Retirada na íntegra de: STOCKER,R & KEANEY JR, JF; **Role of Oxidative Modifications in Atherosclerosis**; *Physiol Rev*; 84: 1381-1478; 2004.

ESTRESSE OXIDATIVO

Estresse oxidativo é o desequilíbrio em que os agentes pró-oxidantes "superam" os agentes antioxidantes. Tal desequilíbrio tem como consequência a oxidação de biomoléculas, o que pode ter consequências danosas, como a alteração da fluidez e integridade de membranas. Um exemplo clássico a esse respeito é a oxidação da cardiolipina, proteína presente nas cristas mitocondriais e que tem papel de estabilização dos complexos da cadeia

transportadora de elétrons, que resulta na liberação de citocromo *c* para o citosol, onde ele estimula vias de sinalização de morte celular (Halliwell and Gutteridge, 1999).

Entre os oxidantes, merecem total atenção os famosos radicais "livres". Radical é o nome dado a átomos e moléculas que possuam um ou mais elétrons desemparelhados (preenchimento ímpar de orbital, ou seja, apenas um elétron ao invés de dois), enquanto radical "livre" é um termo popular e que tem seu uso desaconselhado pela *International Union of Pure and Applied Chemistry* (IUPAC) -http://www.iupac.org/-, a qual estabelece as nomenclaturas em química e também em bioquímica, sendo associada à *International Union of Biochemistry and Molecular Biology* (IUBMB). O exemplo mais clássico de radical é o oxigênio molecular, $^{\cdot}O_2$, um birradical, mais precisamente. No entanto, outros intermediários, não radicais, também apresentam grande importância no estudo e na pesquisa em estresse oxidativo, como o peróxido de hidrogênio, H_2O_2. Dessa forma, as espécies reativas, RS (do inglês *reactive species*), formam o conjunto composto por radicais e por intermediários não radicais. As RS mais conhecidas são as espécies reativas de oxigênio (ROS), mas espécies de outros elementos vêm cada vez mais sendo estudadas, como as espécies reativas de nitrogênio (RNS) (Halliwell and Gutteridge, 1999).

Uma importante fonte de ROS é a cadeia transportadora de elétrons, em que o oxigênio atua como aceptor final de elétrons, sendo reduzido tetravalentemente à água na citocromo *c* oxidase (complexo IV). O complexo IV é o único capaz de reduzir o oxigênio de forma tetravalente, pois em qualquer outro lugar ele será reduzido parcialmente. Quando a cadeia transportadora de elétrons está saturada (em sua atividade máxima) ou inibida, o $^{\cdot}O_2$ passa a aceitar elétrons de outros componentes da cadeia, como o complexo I e a ubiquinona (componente "móvel" da cadeia), originando então o radical ânion superóxido, $^{\cdot}O_2^-$, que mesmo sendo pouco reativo e pouco difusível, está relacionado com vários processos de oxidação. Porém, as principais implicâncias são as reações que podem se originar a partir

dessa última. Com catálise da superóxido dismutase (SOD), o $^{\cdot}O_2^-$ é convertido em H_2O_2, o qual é menos reativo ainda, mas é muito difusível. O peróxido, através das reações de Fenton e de Haber-Weiss, pode dar origem então ao mais potente de todos radicais, $^{\cdot}OH$ (radical hidroxil), extremamente reativo e que pode desencadear severos danos às biomoléculas (Halliwell and Gutteridge, 1999; Nelson et al., 2008; Srinivasan and Avadhani, 2012). Ilustrativamente, podem-se apresentar as ROS formadas de acordo com o nível de redução do oxigênio da seguinte forma (reações não balanceadas estequiometricamente):

$$^{\cdot}O_2 + 1 \text{ elétron} \longrightarrow {}^{\cdot}O_2^-$$

$$^{\cdot}O_2 + 2 \text{ elétrons (ou } {}^{\cdot}O_2^- + 1 \text{ elétron)} \longrightarrow H_2O_2$$

$$^{\cdot}O_2 + 3 \text{ elétrons (ou } H_2O_2 + 1 \text{ elétron)} \longrightarrow {}^{\cdot}OH$$

$$^{\cdot}O_2 + 4 \text{ elétrons (ou } {}^{\cdot}OH + 1 \text{ elétron)} \longrightarrow H_2O$$

Fenton:

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + {}^{\cdot}OH + OH^-$$

Haber-Weiss:

$$Fe^{3+}/Cu^{2+} + {}^{\cdot}O_2^- \longrightarrow Fe^{2+}/Cu^{+} + O_2$$

$$Fe^{2+}/Cu^{+} + H_2O_2 \longrightarrow Fe^{3+}/Cu^{2+} + {}^{\cdot}OH + OH^-$$

Para evitar a formação do radical hidroxil, as células dispõem da enzima catalase (CAT) e de outras enzimas com atividade de peroxidase, tais como glutationa peroxidase (GPx) e peroxiredoxina (Prx).

De uma forma geral, as células possuem um sistema de defesa antioxidante formado por substâncias enzimáticas (como as enzimas citadas acima) e substâncias não enzimáticas. A SOD apresenta, basicamente, três isoformas: SOD-1 (citosólica, que contém cobre e zinco como cofatores), SOD-2 (mitocondrial, que contém manganês como cofator) e SOD-3

(extracelular, assim como a SOD-1 contém cobre e zinco como cofatores), estando essa última intimamente relacionada com a aterosclerose, devido à produção de radical superóxido extracelular pela NADP(H) (nicotinamida adenina dinucleotídeo fosfato) oxidase, NOX, que está muito expressa na membrana dos macrófagos (Fukai and Ushio-Fukai, 2011; Halliwell and Gutteridge, 1999). A CAT está presente nos peroxissomos e tem um alto Km, enquanto a GPx está presente no citosol e tem um Km menor. A GPx usa um cofator como agente redutor, o tripeptídeo glutationa (GSH), que apresenta o aminoácido cisteína em sua estrutura, o qual possui o grupo redox-ativo sulfidril (-SH) em sua cadeia lateral. Na reação, duas moléculas de glutationa são oxidadas, sendo formada uma ligação covalente entre elas, chamada "ligação dissulfeto", originando o dissulfeto de glutationa (GSSG). Para garantir a eficiência do sistema, o GSSG é reduzido à GSH por catálise da glutationa redutase (GR), que usa o cofator NADPH como agente redutor. Similarmente, a Prx catalisa a remoção do peróxido de hidrogênio, sendo ela própria o agente redutor, e sua redução depende do cofator tiorredoxina (Trx). O estado reduzido da Trx é mantido por catálise da Trx redutase (TrxR), usando NADPH como agente redutor (Halliwell and Gutteridge, 1999; Park et al., 2014; Park and Oh, 2011). O sistema não enzimático inclui várias substâncias, entre elas a própria GSH, a albumina, os compostos fenólicos, a vitamina A, a vitamina C, a vitamina E e outras. A GSH está presente em grande quantidade nos citoplasmas e é considerada como o tampão redox intracelular, enquanto a albumina (que contém várias cisteínas ao longo de sua estrutura) é considerada como o tampão redox plasmático. A vitamina A representa, na verdade, uma classe de substâncias que apresentam atividade de retinol. Fontes alimentares de vitamina A são os carotenoides presentes em vegetais. Além disso, a vitamina A, assim como a vitamina E, é um composto lipofílico, enquanto a vitamina C é um antioxidante hidrofílico (Farbstein et al., 2010; Halliwell and Gutteridge, 1999).

Desde a descrição do ˙NO na década de 80, o interesse pelas RNS cresceu consideravelmente. Três isoformas da enzima responsável pela catálise da síntese do ˙NO são descritas com mais frequência, a óxido nítrico sintase endotelial constituinte (eNOS), a óxido nítrico sintase neuronal constituinte (nNOS) e a óxido nítrico sintase induzível (iNOS). Apesar dos benéficos efeitos fisiológicos descritos para o ˙NO na parede vascular, sua produção desregulada pode ter consequências danosas. O ˙NO pode reagir com o $\dot{O}_2^-$, gerando o $ONOO^-$ (peroxinitrito), o qual pode modificar proteínas por nitrar os resíduos tirosil delas (formação de 3-nitrotirosina). Esse tipo de dano, oriundo de um "estresse nitrosativo", vem sendo largamente estudado e associado a uma série de doenças e distúrbios, inclusive aterosclerose (Halliwell and Gutteridge, 1999; Ignarro, 2002; Peluffo and Radi, 2007).

MACRÓFAGOS

Como discutido anteriormente, os macrófagos possuem um papel central no desenvolvimento da aterosclerose, devido à sua capacidade de fagocitar oxLDL e de responder à ela com o agravamento do processo inflamatório. No entanto, recentemente vem sendo discutido o efeito dos diferentes fenótipos no desenvolvimento de doenças, pois dois principais fenótipos são descritos, o fenótipo classicamente ativado, M1 (com característica pró-inflamatória), e o fenótipo alternativamente ativado, M2 (com característica anti-inflamatória). Sendo a aterosclerose considerada uma doença inflamatória, torna-se intrigante a compreensão de como o fenótipo dos macrófagos pode ser modulado (Parthasarathy et al., 2010; Shimada, 2009).

Recentemente, nosso grupo de pesquisa publicou um artigo de revisão que descreve a relação dos diferentes tipos de macrófagos com câncer de pulmão de não pequenas células, NSCLC (do inglês *non-small cell lung cancer*). De fato, muitos estudos vêm sendo

conduzidos na área do câncer a fim de compreender a polarização existente entre os dois fenótipos, sendo o M1 associado ao combate ao tumor, enquanto o M2 está associado com a progressão tumoral. *In vitro* o fenótipo M1 pode ser induzido a partir do tratamento com interferon gama (IFN-γ) em associação com lipopolissacarídeo bacteriano (LPS) e/ou fator de necrose tumoral alfa (TNF-α), e é caracterizado pela expressão gênica de TNF-α, interleucina 1 (IL-1), IL-12 e iNOS. Já o fenótipo M2 pode ser induzido *in vitro* por tratamento com IL-4, IL-10 ou IL-13 e se caracteriza pela expressão gênica de fator de crescimento endotelial vascular, VEGF (do inglês *vascular endothelial growth factor*), e de fator de transformação do crescimento beta, TGF-β (do inglês *transforming growth factor beta*) (Becker et al., 2014).

BIOINFORMÁTICA

Diferentes estratégias de pesquisa básica vêm contribuindo para o desenvolvimento do conhecimento em ciências biológicas e da saúde, como os clássicos modelos experimentais *in vitro*, *ex vivo* e *in vivo*. No entanto, cada vez mais o modelo não experimental (teórico), já bem consolidado em áreas como física e química (física e química teóricas/computacionais), vem ganhando espaço nas ciências biológicas e da saúde, sendo um forte aliado para otimizar os experimentos de bancada. De uma forma geral, as simulações computacionais são chamadas de análises *in silico*.

A bioinformática, também conhecida como biologia teórica/computacional, pode ser dividida em duas grandes áreas, a bioinformática estrutural e a bioinformática não estrutural (tradicional ou clássica), sendo a primeira intimamente relacionada à química teórica. Através da bioinformática estrutural é possível realizar simulações de estrutura e de conformação de biomoléculas, bem como interações que elas podem efetuar, pelos processos de modelagem e

dinâmica molecular, sendo essa ferramenta muito útil, por exemplo, para o desenvolvimento racional de fármacos.

Por outro lado, a biologia de sistemas (importante campo de estudo da bioinformática não estrutural) se propõe a fazer uma análise considerando a complexidade de um sistema, ou seja, avaliar "o todo". A partir do início deste século, a área teve um grande crescimento, estimulado, por exemplo, pelo aumento na obtenção de dados "ômicos". Para o desenvolvimento das análises, a biologia de sistemas estabelece as interelações de partes individuais de um sistema, a partir da construção gráfica de conjuntos de nós e conectores. Cada nó representa uma unidade, como uma proteína, por exemplo, enquanto o conector representa o grau de associação entre pares de nós, formando então uma rede de interação. Diversos tipos de redes podem ser exemplificados, desde uma rede de interação gênica à *World Wide Web*, uma das maiores redes de comunicação no mundo. Frequentemente o termo rede é usado como sinônimo de grafos, mas, na verdade, um grafo descreve a análise matemática de uma rede, enquanto a rede descreve as interações funcionais do sistema em questão (Verli, 2014).

JUSTIFICATIVA

A aterosclerose, assim como o diabetes, é uma doença metabólica que acomete grande parte da população mundial, estando associada a fatores genéticos e também ambientais, tais como a má alimentação ("dieta de cafeteria") e o sedentarismo. Aliás, de acordo com a Organização Mundial da Saúde, WHO (do inglês *World Health Organization*) - http://www.who.int/en/-, as doenças cardiovasculares são responsáveis por aproximadamente 30% das mortes no mundo. Apesar de haver uma grande quantidade de trabalhos de pesquisa em torno dessa doença, muitas lacunas ainda carecem de um melhor entendimento. Um aspecto intrigante é o fato de intervenções antioxidantes, propostas devido à forte influência do estresse oxidativo na patogenia da aterosclerose, apresentarem resultados muito controversos.

Existem diferentes abordagens para uma investigação científica, como os experimentos *in vitro*, *ex vivo* e *in vivo*. Além desses, cada vez mais os experimentos *in silico* (ciência computacional) vêm se tornando grandes aliados nas estratégias de pesquisa. A Bioinformática, utilizada no presente trabalho, possibilita a análise de grandes bancos de dados (de difícil análise por ferramentas "tradicionais" de computação), a simulação de interações biológicas (como a interação entre um fármaco e seu receptor) e um melhor direcionamento nos experimentos de bancada (experimentos práticos), um menor custo e uma grande interação entre pesquisadores de diversos locais, já que se trabalha com bancos de dados de livre acesso.

OBJETIVO GERAL

CAPÍTULO I

Revisar, a partir de dados apresentados na literatura, o papel dos antioxidantes e dos fenótipos de macrófagos na aterosclerose.

CAPÍTULO II

Comparar as expressões de grupos de genes (antioxidantes, relacionados ao fenótipo M1 e relacionados ao fenótipo M2) entre placas de ateroma em estágio inicial e placas de ateroma em estágio avançado, e entre macrófagos de pessoas saudáveis e macrófagos de pessoas com aterosclerose.

OBJETIVOS ESPECÍFICOS

CAPÍTULO I

- Revisar, a partir de dados apresentados na literatura, o papel dos oxidantes na aterosclerose;
- Revisar, a partir de dados apresentados na literatura, o papel das terapias antioxidantes na aterosclerose;
- Revisar, a partir de dados apresentados na literatura, o papel dos fenótipos M1 e M2 de macrófagos na aterosclerose;
- Revisar, a partir de dados apresentados na literatura, o papel da oxidação de lipoproteínas de baixa densidade (LDL) na aterosclerose;

CAPÍTULO II

- Comparar as expressões de grupos de genes (genes antioxidantes, genes relacionados ao fenótipo M1 e genes relacionados ao fenótipo M2) entre macrófagos de pessoas com aterosclerose e macrófagos de pessoas saudáveis;
- Comparar as expressões de grupos de genes (genes antioxidantes, genes relacionados ao fenótipo M1 e genes relacionados ao fenótipo M2) entre *foam cells* de pessoas com aterosclerose e *foam cells* de pessoas saudáveis;
- Comparar as expressões de grupos de genes (genes antioxidantes, genes relacionados ao fenótipo M1 e genes relacionados ao fenótipo M2) entre *foam cells* de pessoas saudáveis e macrófagos de pessoas saudáveis;
- Comparar as expressões de grupos de genes (genes antioxidantes, genes relacionados ao fenótipo M1 e genes relacionados ao fenótipo M2) entre *foam cells* de pessoas com aterosclerose e macrófagos de pessoas com aterosclerose;

- Comparar as expressões de grupos de genes (genes antioxidantes, genes relacionados ao fenótipo M1 e genes relacionados ao fenótipo M2) entre placas ateroscleróticas humanas em estágio avançado e placas ateroscleróticas humanas em estágio inicial;

PARTE II

CAPÍTULO I

The role of different oxidants on oxLDL formation and the possible impact on macrophage phenotype in atherosclerosis

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The role of different physiological oxidants on oxLDL formation and the potential impact on macrophage's polarization phenotype: a novel therapeutic venue for atherosclerosis?

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Abstract

Atherosclerosis is a pro-inflammatory disease involved with a systemic oxidative stress status. Some general pathophysiological features of this disease are such as endothelial dysfunction, low density lipoprotein (LDL) oxidation, macrophage-mediated inflammation, foam cells formation, smooth muscular cells (SMC) dysfunction and migration, atheroma plaque formation and plaque rupture. However, until now, it is difficult to determine the trigger event disease. It is well-known that oxidized LDL (oxLDL) is recognized by a class of specific macrophage's membrane receptor and, once the lipoprotein is internalized, a pro-inflammatory signal is initiated. However, two macrophages (Mϕ) phenotypes are present in atherosclerosis region, a pro-inflammatory M1 (classically activated) and an anti-inflammatory M2 (alternatively activated), but the specific role played by M1/M2 Mϕ phenotypes remains to be better cleared. Additionally, it has been suggested that different oxidants and oxidants products may impact directly the general pathophysiological features of atherosclerosis: oxLDL and Mϕ. Furthermore, the results regarding antioxidant treatments are very conflicting. Therefore, herein we review the basic mechanisms of oxidative stress and the available literature on the antioxidant therapies, the role of macrophages in atherosclerosis and general oxidative modifications of LDL molecules.

Key Words

Antioxidants, atherosclerosis, foam cell, free radicals, oxidants, oxLDL.

INTRODUCTION

Atherosclerosis is a pro-inflammatory disease characterized by endothelial dysfunction, vascular macrophage infiltration and lipids accumulation. However, it is not a simple process of fats deposition, actually, a sophisticated and complex mechanism is involved on the pathogenesis [1]. It is well-know the existence of a disturbance on endothelium homeostasis, which is physiologically controlled mainly by nitric oxide production, a radical molecule responsible to the smooth muscular cell relaxation (vasodilatation). Nitric oxide exerts its effects by diffusing into smooth muscular cell (the media, where smooth muscular cells are distributed in a multicellular layer) through guanylate cyclase oxidation and activation [2].

When endothelium is damaged, it loses its physiological properties and, in addition, a pro-inflammatory process is initiated [1]. Many coronary risk factors are associated with endothelial dysfunction (ED), such as hypercholesterolemia, hypertension, smoking and diabetes [3]. Oxidative stress and inflammation are among the mainly mechanisms underlying the ED [4]. Plasmatic low density lipoprotein (LDL) infiltrates into subintima region where can be oxidized by different free radicals or their related reactive species (RS), yielding oxLDL (oxidized LDL). Macrophages (activated monocytes present in the tissue) and smooth muscular cells (SMC) present receptors that recognize oxLDL and this interaction initiate a NFκB (nuclear factor kappa B)-mediated inflammatory response. Therefore, more macrophages are attracted to subintima region and the SMC lose their physiological characteristic and obtain an inflammatory macrophage-like phenotype. In addition to the ability to bind membrane receptors, oxLDL are phagocyted by macrophages, which have their volumes increased and acquire the foam cell characteristic. Moreover, macrophages and SMC secret matrix metalloproteinases (MMP), enzymes capable to degrade extracellular matrix, what allows the SMC migration to intima [1, 5-7]. Thus, SMC migrated, excess of macrophages and the increased macrophages volume, all together combined with endothelial dysfunction yield the atheroma plaque, diminishing the vessel light and increasing

the blood pressure. Thereafter, the plaque can disrupt, releasing a thrombus into the bloodstream (thrombosis), which can block the tissue blood flow in a smaller caliber artery, as the coronary, the artery responsible for heart feeding, generating the coronary heart disease [1, 8].

A novel perspective has instigated the researchers: the macrophage phenotype. Indeed, macrophages can present two phenotypes: a pro-inflammatory (M1) and an anti-inflammatory (M2). Regarding the inflammatory feature of atherosclerosis, it is reasonable think that M1 could be the phenotype present in the disease [9]. Additionally, LDL can be oxidized by different agents, generating different modifications [10]. Thereby, three questions can be postulated: 1. What is the macrophage phenotype in atherosclerosis? 2. What signal induces each phenotype in atherosclerosis? 3. Can "different" oxLDL molecules (oxidized by different oxidants) induce a specific macrophage phenotype? Therefore, our aim in the present work is to review about the role of macrophage phenotypes, LDL oxidation and therapeutic antioxidant strategies on atherosclerosis.

REACTIVE SPECIES AND OXIDATIVE STRESS

According to IUPAC (International Union of Pure and Applied Chemistry), a radical is a chemical entity containing one or more unpaired electrons (each orbital is occupied by only one electron). The "free" radical name is popularly known, but it is not recommended by IUPAC (http://www.iupac.org/). A largely known radical is the molecular oxygen, which is a biradical molecule. A radical can be reduced by oxidizing another chemical entity. However, many of these chemical entities can be important structural and/or functional molecules, such as lipids, proteins, carbohydrates and nucleic acids, which are essentials to life cells homeostasis. Actually, many diseases and dysfunctions are associated to uncontrolled radicals production, mainly by their capacity to damage biomolecules. Nevertheless, there are substances that can react with the oxidants radicals, neutralizing them, known as antioxidants [11]. An important antioxidant is the ascorbic acid, the vitamin c, which was of great interest for the very important chemist Dr. Linus

Carl Pauling [12]. Oxidative stress is named the imbalance between oxidants and antioxidant species, which can generate oxidative damage [11].

Radicals and their related species (non-radicals) are called as reactive species (RS) and represent a lot of interest to many researchers in the world. A special and largely studied class of RS is the reactive oxygen species (ROS). The main physiological source of ROS is the mitochondrial respiratory chain. It is well-known that oxygen molecule is reduced to water by receiving four electrons (tetravalent reduction) at cytochrome *c* oxidase complex (IV complex) of the respiratory chain. However, IV complex is the only one able to reduce oxygen in a tetravalent way, and when oxygen is reduced in another respiratory chain site (as the I complex) it is partially reduced, generating ROS involved with many physiological dysfunctions. Monovalent reduction of oxygen gives origin to the superoxide radical, which can also be reduced in a monovalent way yielding hydrogen peroxide, a non-radical molecule. Hydrogen peroxide is very stable and can across membranes, but if it suffers monovalent reduction and hydroxyl radical is formed, the most potent and dangerous radical [11, 13, 14].

There are enzymatic and non-enzymatic antioxidants to deal with ROS. Superoxide is removed by superoxide dismutase (SOD) reaction, yielding hydrogen peroxide as product. Human cells play three SOD isoenzymes: SOD-1 (cytosolic, presents copper and zinc as cofactors); SOD-2 (mitochondrial, presents manganese as cofactor); SOD-3 (extra-cellular, presents copper and zinc as cofactors) [11, 15]. Two important reactions can drive hydrogen peroxide to generate hydroxyl, the Haber-Weiss and the Fenton reactions. To avoid hydroxyl synthesis, catalase (hydroperoxidase) enzyme can convert hydrogen peroxide into water. Besides it, glutathione peroxidase (GPx) also catalyzes the conversion of hydrogen peroxide into water. GPx needs two glutathione (GSH) molecules as reducing cofactors, reducing them to glutathione disulfide (GSSG); Glutathione reductase (GR) uses reduced nicotinamide adenine phosphate (NADPH) to recover the levels of GHS. Similarly, peroxiredoxin convert hydrogen peroxide into water (peroxiredoxin is oxidized in this process) and, thereafter, use thioredoxin (Trx) molecule to recharge itself (peroxiredoxin

reduction) [11, 16, 17]. Non-enzymatic system presents hydrophilic substances, as vitamin C (ascorbic acid), GSH and uric acid. Moreover, lipophilic substances also exert non-enzymatic antioxidant actions, such as vitamin E (α-tocopherol) and vitamin A. Actually, vitamin A is represented by a large number of molecules that play a function of retinol molecule. In addition to retinol (and its acid form, retinoic acid), there are the carotenoids molecules, which are synthesized by vegetable cells [11, 18].

Another class of molecules that represent a great interest in chemistry and medicine is the reactive nitrogen species (RNS) class. The first described RNS was the radical nitric oxide, discovery that ensured the Physiology and Medicine Nobel Prize of 1998 to three researchers: Ferid Murad, Robert F. Furchgott and Louis Ignarro. Nitric oxide plays an important role on vascular physiology and is synthesized by nitric oxide synthase (NOS) catalysis. Another relevant RNS is the non-radical peroxynitrite, generated by the reaction between nitric oxide and superoxide radicals. Peroxynitrite can modify molecules by nitration, leading to their dysfunction. In proteins, the modification is very specific, being performed at tyrosil residue, resulting in the 3-nitrotyrosine modification [2, 11, 19].

OXIDANTS AND ATHEROSCLEROSIS

Nitric oxide synthase (NOS) produces nitric oxide in physiological status, but, depending on cofactors and the environment, it can produce superoxide radical. The genetic deletion of endothelial NOS ($eNOS^{-/-}$) in apolipoprotein E-knockout mice ($apoE^{-/-}$) increases the interaction of leucocytes and endothelium, analyzed by intravital microscopy. Additionally, vascular superoxide production is higher in $apoE^{-/-}$ than $apoE^{-/-}/eNOS^{-/-}$ mice [20]. Double-knockout mice for $apoE^{-/-}/eNOS^{-/-}$, in a Western-type diet-fed, played increased atherosclerosis lesion compared to "Western-type" (fats represent 42% of the total calories) diet-fed $apoE^{-/-}$ mice [21]. Sentman *et al* showed that extracelular superoxide dismutase (SOD) activity has little effects on atherosclerosis in mice [22].

Asynmetrical dimethylarginine (ADMA), an inhibitor of eNOS, has been considered as a pro-atherogenic agent. It was published an interesting study that evaluated the association of ADMA and eNOS. Patients undergoing coronary bypass surgery (n = 201) had withdrawn their serum and paired samples of saphenous vein and internal mammary arteries. The research work showed an association between elevated serum ADMA concentration and total superoxide production by eNOS uncoupling [23]. Tetrahydrobiopterin treatment reversed the atherogenic effects generated by apoE$^{-/-}$ and high-fat diet in mice [24]. Recently, Antoniades *et al* also published an interesting study with patients undergoing coronary artery bypass graft surgery. It was observed that statins reduce vascular superoxide production and ameliorates the bioavailability of nitric oxide through an uncoupling of NOS [25]. Another work suggests an addictive action of nitric oxide and statins. The use of a nitric oxide donor potentiated the anti-inflammatory and anti-atherogenic effects of statin in a model of mice with low density lipoprotein receptor (LDLR) deletion, LDLR$^{-/-}$, fed with a high-fat diet [26]. The interaction between superoxide and nitric oxide can generate a potent nitration agent, peroxynitrite, and diminish the nitric oxide availability in atherosclerosis, as suggested by a study that has used a cholesterol-fed rabbit model [27]. Actually, nitric oxide donors can to suppress the superoxide production via inhibition of endothelial nicotinamide adenine dinucleotide phosphate (NADPH) oxidases by subunit p47phox S-nitrosilation [28]. Sorescu *et al* investigated the superoxide production localization in atherosclerotic arteries as well as the expression of the NADPH oxidases (NOX) family's enzymes (gp91phox, Nox1 and Nox4). Samples (coronary arteries) were removed from patients undergoing heart transplantation. In non-atherosclerotic arteries, superoxide production was homogenous in intima, media and adventitia, while in atherosclerotic arteries was presented an increased production on plaque shoulder. Nox1 and gp91phox were associated with intracellular oxidative stress and may be related to severity of atherosclerosis [29]. Atherosclerotic lesion and superoxide production are decreased in apoE$^{-/-}$ mice with nonfunctional monocyte/macrophage NADPH oxidase (BMO) and apoE$^{-/-}$ mice with nonfunctional vessel/wall NADPH oxidase (VWO) when both are compared to apoE$^{-/-}$ mice

(control) [30]. Interestingly, it was demonstrated, *in vivo* with in an apoE$^{-/-}$ mice model, co-localization of 3-nitrotyrosine with endoplasmic reticulum (ER) stress in early atherosclerosis lesions. Additionally, the same study played *in vitro* (cultured endothelial cells) that peroxynitrite induced depletion of cytosolic calcium, a known mechanism of ER [31]. It has been suggested that the protein nitration can be the mediator of the CD40 and its ligand effects on immune response in atherosclerosis. Cultured human aortic endothelial cell exposed to clinical concentrations of CD40 ligands played decreased nitric oxide and superoxide bioactivity and increased prostacyclin synthase nitration. Moreover, the administration of CD40 ligands in C57BL6 mice resulted in nitration and inhibition of aortic prostacyclin synthase. However, the CD40 ligands administration in mice overexpressing superoxide dismutase induced a lesser nitration and inhibition of prostacyclin synthase, indicating the peroxynitrite role [32]. Moreover, nitric oxide can act as a heme oxygenase (HO) enzyme modulator. The HO catalyzes the heme (a pro-oxidant agent) degradation into carbon monoxide, iron and bilirubin (an antioxidant agent). It was reported that nitric oxide production is decreased in a model using atherosclerotic New Zealand rabbits. The HO expression is increased in a low rate, probably as an adaptation response to hypercholesterolaemia. However, with statins treatment, the nitric oxide production was partially restored and that increased HO expression played a further increasing [33]. It is well-known the role of matrix metalloproteinases (MMP) in atherosclerosis development. Zalba *et al* demonstrated that superoxide radical stimulates MMP-9 secretion and activity by *in vitro* model using human monocytes [34].

Peroxynitrite and hydrogen peroxide were associated with mitochondrial deoxyribonucleic acid (DNA) damage in vascular cells. Reactive species presented a stronger damage in endothelial cell than in smooth muscular cells, while peroxynitrite inhibited the mitochondrial protein synthesis in a dose-dependent way, resulting in diminished adenosine triphosphate (ATP) levels [35]. ApoE$^{-/-}$ mice overexpressing alone catalase or both catalase and Cu/Zn-SOD presented reduced atherosclerotic lesions and F2-isoprostanes levels in plasma and aorta when compared to ApoE$^{-/-}$

mice (control). The results for ApoE$^{-/-}$ mice overexpressing alone catalase were similar to those for control. Authors suggest that hydrogen peroxide could play a higher role in atherosclerosis than superoxide [36]. The deficiency of glutathione peroxidase, important enzyme on hydrogen peroxide removal, is associated with atherosclerosis progression and modifications of lesions in a model of apolipoprotein E-deficient mice [37]. The role of hydrogen peroxide in atherosclerosis seems to be related to the involvement of homocysteine with this disease. Researchers from Japan demonstrated, in a cell culture model vascular smooth muscular cell (VSMC) from rat aorta artery, the ability of homocysteine to potentiate the mitogenic effect of platelet-derived growth factor-BB (PDGF-BB), possibly due the redox imbalance, since homocysteine treatment yields increased superoxide dismutase (SOD) activity and decreased glutathione peroxidase (GPx) activity [38]. However, endogenous hydrogen peroxide plays an important physiologic role in acetylcholine-dependent vasorelaxation [39]. The impairment on neuronal nitric oxide synthase (nNOS)-derived hydrogen peroxide can contribute to endothelial dysfunction in aorta from apolipoprotein E-knockout mice (apoE$^{-/-}$) [40]. It was suggested by Jaimes *et al* in 2001, in endothelium cells from porcine aorta, that hydrogen peroxide affects nitric oxide production via receptor-dependent and receptor independent agonists mechanisms, in part, by eNOS cofactors inactivation. The same study showed that hypoclorite also impairs nitric oxide production, but only via receptor-operated mechanisms [41]. Xu *et al* have proposed another mechanism for hypoclorous acid action (HOCl). It would uncouple eNOS via peroxynitrite derived from PKC-ζ-dependent NADPH oxidase [42]. Hydrogen peroxide has been associated to play a central role on cell proliferation signaling [43]. Pachenko *et al* studied arterial segments collected from human autopsies and have found that heterogeneous nuclear ribonucleoprotein (hnRNP-C), a hydrogen peroxide-activated nuclear pre-mRNA binding protein that is involved with cell proliferation and differentiation, is upregulated in atherosclerotic and pre-atherosclerotic lesions [44]. Analysis in abdominal aorta arteries of human cadavers concluded that atherosclerotic plaques have a high pro-oxidant environment, since all of these

samples induced lipoperoxidation in rat liver microsomes, what was inhibited by desferrioxamine, an iron-ion (promoter of hydroxyl radical production) chelator [45].

THE ROLE OF MACROPHAGES

Recently, our research group published a review article elucidating the role of different macrophage phenotypes and their relation with non-small cell lung cancer (NSCLC). In cancer, there is a macrophage polarization, being the M1 (classically activated) a pro-inflammatory and cytotoxic phenotype acting against tumor cells. The M2 (alternatively activated) is an anti-inflammatory phenotype and is associated with the tumor progression. *In vitro* induction of M1 is performed with interferon (IFN)-γ treatment, in combination with lipopolissacharide (LPS) and/or tumor necrosis factor (TNF)-α, while M2 can be modulated by interleukin (IL)-4, IL-10 and IL-13. About gene expression, we can see TNF-α, IL-1, IL-12 and inducible NOS (iNOS) in the M1 phenotype. On the other hand, vascular endothelial growth factor (VEGF) and transforming growth factor (TGF)-β being expressed by M2 phenotype [66].

The pro-atherogenic hsCRP induced macrophages to M1 phenotype both *in vitro* and *in vivo* (rats), and, in addition, M2 (monocytes primed with IL-4) were converted into M1 *in vitro* by hsCRP treatment [67]. The antioxidant molecule Trx has effects on macrophage differentiation, promoting the IL-4/IL-13-induced M2 and reducing LPS-induced M1 differentiations *in vitro*. Equivalent effects were found in hyperlipoproteinemic ApoE2.Ki mice challenged with IL-4 or LPS. The LPS-challenged mice played severe atherosclerotic lesion with increased M1 over M2. Trx shifted the phenotype patern and, besides it, diminished the lesion area [68]. Additionally, it is well-known the angiotensin II is determinant in atherosclerosis and that macrophages (the main cell type in atheroma plaques) express angiotensin II type 1 receptor (AT1). Yamamoto et al concluded that AT1 exerts its atherogenic effects through M1 phenotype induction [69].

It was demonstrated that the adiponectin molecule was able to drive monocytes into the alternative activation M2 [70]. The IL-13 administration induced M2 phenotype in atherosclerosis-prone mice. Additionally, the M2 macrophage was more efficient to hand with lipid than IFN-γ-activated M1 [71]. Interestingly, Oh et al took important conclusions from their experimental model. Macrophages from diabetic subjects were stimulated to M1 or M2 and, then, treated with oxLDL. The induction of scavenger receptors CD36 or SR-A1 resulted in an increased foam cell formation by M2 stimulation. Moreover, it is mediated by endoplasmic reticulum (ER) stress [72]. Recently, the same research group demonstrated that vitamin D is a natural reliever of ER stress and could be an atheroprotective agent [73].

In vitro determinations of arginase (Arg) expression showed that Arg II expression is associated with M1 phenotype, but Arg I is associated with the M2. By the use of this information, it was possible identify, *in vivo* (model of $ApoE^{-/-}$ mice), the plasticity of the macrophages in atherosclerotic lesions. In early lesions, M2 is predominant, contributing to *in vitro* SMC proliferation. In aged mice, M1 is predominant, favoring the atherosclerosis progress. It is hypothesized there is a M2 -> M1 switch in the lesion area and that strategies to avoid or revert it could be a good atheroprotective tool [74].

Hirose *et al* performed a very interesting study, which they evaluated the effect of oxLDL on gene expression of M0 (non-polarized macrophage), M1 and M2. It was observed that oxLDL uptake can interfere on M1 function through TGF-β1 and NFκB, but not on M0 and M2 [75]. Isa et al showed that M2 are more susceptible to lipotoxicity induced by oxLDL [76]. Macrophages treated with oxidized phospholipids drive into a different phenotype, Mox (different from M1 and M2, presenting lower phagocytotic and chemotactic capacity). Mox is characterized by a higher expression of redox-regulatory genes, which are mediated by the transcription factor Nrf2, and represent about 30% of macrophages in atherosclerotic lesion area of $LDLR^{-/-}$ mice [77]. It was demonstrated that heme group drives macrophage to an unknown atheroprotective macrophage phenotype called Mhem, which express activating transcription factor (ATF)-1 [78].

IMPLICATIONS OF OXIDIZED LOW DENSITY LIPOPROTEIN (oxLDL)

Agil *et al* demonstrated that LDL is oxidized (lipi peroxides formation) by ferrous ion and hydrogen peroxide system, but not by one of them alone, suggesting a Fenton reaction mechanism [79]. Superoxide does not increase the oxidation level of LDL, as well as does not delay this process [80, 81].

Nitric oxide can act as an antioxidant for LDL, based on its ability to readily traverse the surface towards the hydrophobic lipid core [82]. The oxidative damage induced by peroxynitrite in LDL is inhibited by metallothionein [83]. Protein nitration can contribute to the LDL apolipoprotein (Apo) B-100 unfolding [84]. LDL isolated from human aortic atherosclerotic intima had high levels of 3-nitrotyrosine [85]. Additionally, RNS can be generated from mieloperoxidase activity. LDL incubation with mieloperoxidase, hydrogen peroxide and nitrite (NO_2^-) resulted in nitration of tyrosil residues from Apo B-100 and the beginning of lipid peroxidation. The modified LDL induced a foam cell formation from macrophages [86].

Hypochloride reacts with LDL Apo B-100 targets, oxidizing them. Additionally, vitamin C can protects from this hypochloride-induced damage [87]. However, Hazell et al suggest that the reaction of hypochloride with Apo B-100 yields secondary radicals, which could be responsible for extended LDL lipid peroxidation [88]. Interestingly, LDL submitted to a system formed by mieloperoxidase + hydrogen peroxide + ion chloride (Cl^-) at an acidic pH generated chlorinated sterols, suggesting a role for HOCl (hypochlorous acid) [see eq. 1]. Nevertheless, in an Cl^- —free environment, HOCl was not able to produce the same LDL chlorinated sterols, indicating a role for molecular chlorine (Cl_2) [see eq. 2]. Thus, it was proposed that the mieloperoxidase effects are mediated by Cl_2 instead HOCl [89].

$$Cl^- + H_2O_2 + H^+ \longrightarrow HOCl + H_2O \quad \text{(Eq. 1)}$$

$$HOCl + H^+ + Cl^- \longrightarrow Cl_2 + H_2O \quad \text{(Eq. 2)}$$

ANTIOXIDANT THERAPIES IN ATHEROSCLEROSIS

Shimada *et al* demonstrated that the antioxidant N-acetylcysteine (NAC), administrated with intraperitoneal injection for eight weeks (three times per week), is able to reduce some atherosclerotic endpoint in animal models. Apo E-deficient mice were fed with a high-fat diet containing 0.3% cholesterol and 20% fat for two weeks, and, thereafter, were submitted to the NAC treatment. The antioxidant treatment decreased atherosclerotic lesion area and the superoxide production. However, no changes on lipids profile were found [46]. Six months of supplementation with zinc increased its plasmatic concentration in elderly subjects. The elevated plasmatic zinc concentration was inversely proportional to plasmatic levels of high-sensitivity C-reactive protein (hsCRP), interleukin (IL)-6, macrophage chemoattractant protein 1 (MCP-1), vascular cell adhesion molecule 1 (VCAM-1), secretory phospholipase A2, and malondialdehyde and hydroxyalkenals (MDA + HAE) [47]. Iron chelation, through deferoxamine injection, was able to improve the blood flow in patients with CAD [48].

The Vitamin E Atherosclerosis Prevention Study (VEAPS) followed more than 300 patients for approximately 3 years receiving DL-α-tocopherol (400 IU per day) or placebo. Even with elevated plasmatic vitamin E and diminished circulating oxLDL, the treatment was not able to reduce the progression of intima-media thickness (IMT) [49]. The effect of RRR-α-tocopherol supplementation (1200 IU/day) was evaluated in 90 patients with coronary artery disease (CAD) for 2 years, in a randomized, controlled, double-bind trial. It was presented that the intervention increased plasmatic α-tocopherol and decreased plasmatic hsCRP and urinary F2-isoprostanes [50]. The Los Angeles Atherosclerosis Study (cohort of 573 middle-aged subjects) suggested that elevated plasma levels of oxygenated carotenoids (lutein, β-cryptoxanthin and zeaxanthin) and α-carotene can present an important role for protecting against early atherosclerosis [51]. Patients with CAD received a single-dose (2g PO) and 500mg/day for 30 days (long-term) of vitamin C (in a

randomized, double-blind, placebo controlled-study). Plasmatic vitamin C concentration and flow-mediated dilation (FMD) increased after single-dose and were sustained after long-term, while no differences were observed on placebo [52]. The *Endothelial Assessment of Risk from Lipids in Youth* (EARLY) Trial (a randomized, double-blind, placebo controlled-trial) presented improvement on endothelium-dependent FMD of the brachial artery in children with familial hypercholesterolemia after 6 weeks under vitamins C and E supplementation. However, no differences were found regarding LDL oxidation, F2-isoprostanes, 8-hydroxy-2'-deoxyguanosine and hsCRP [53]. In addition, the *Antioxidant Supplementation in Atherosclerosis Prevention* (ASAP) study found a positive effect of vitamins C and E combination, since they can slow down the progression of atherosclerosis in hypercholesterolemic people [54]. A population-based study, which followed 840 middle-aged men from eastern Finland for seven years, concluded that high serum levels of carotenoids can be protective against early atherosclerosis, since IMT was inversely correlated with lycopene, α-carotene and β-carotene [55].

Coenzyme Q supplementation was associated with reduction in oxidative damage of DNA [56]. The supplementation with $20mg.dL^{-1}$ lutein capsules reduced serum inflammatory cytokines in atherosclerosis patients associated with its serum increasing [57]. Chronic decaffeinated green tea extract (catechins tablet) supplementation reduced reactive oxygen species (ROS) production and proinflammatory cytokines in end-stage renal disease patients undergoing hemodialysis [58]. After two weeks of green tea drinking, the blood was collected from the five women enrolled in the study. It was observed in the plasma an increase in conjugated dienes formation, but a decrease in cholesterol content. No changes were detected in β-carotene, α-tocopherol, vitamin c and uric acid contents and SOD activity [59]. Treatment with catechin extracted from green tea of leaves decreased plasma oxLDL in healthy adults, but had no effect plasma lipids concentrations [60]. High polyphenol content diet was better than low polyphenol content diet to healthy subjects, since it reduced the LDL oxidation and the CD40L gene expression [61].

Patients with metabolic syndrome had decreased their plasmatic levels of total and LDL-cholesterol and VCAM-1 after eight weeks under strawberry supplementation [62]. Patients with CAD that were treated for eight weeks with 200mg/day of pycnogenol (a proprietary bark extract of the French maritime pine tree) had an improvement on FMD and decreased level of F2-isoprostanes. No changes on inflammation and blood pressure [63]. The consumption of flavonoids through diet with onion and black tea presented no effect on plasma F2-isoprostanes and malondialdehyde-LDL autoantibody titer [64]. However, the consumption of soy containing isoflavone phytoestrogens generated reduced plasma F2-isoprostanes [65].

CONCLUSION

Cardiovascular pathologies are of great interest of many researchers, since these diseases are responsible for around 30% of deaths in the world, http://www.who.int/en/. The complexes mechanisms about atherosclerosis still need more explanations to achieve better therapeutic strategies. The role of macrophage phenotype and the LDL oxidation can be promising targets to health sciences. Actually, the two macrophages phenotypes (M1 and M2) are present in atherosclerosis development, as well as other activation profiles (like Mox and Mhem), and it is very important the comprehension of their roles and action mechanisms. Moreover, in addition to conflicting results regarding antioxidant therapies, it has been described the different ways to oxidize the LDL macromolecule, but the impact of these modifications on the role of oxLDL as a cell function modulator remains to be explored.

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FIGURE CAPTIONS

Figure 1: LDL oxidation by different oxidants, such as radical anion superoxide, hydrogen peroxide, radical anion hydroxyl, radical nitric oxide, peroxynitrite and hypochlorous acid;

Figure 2: The macrophage (Mϕ) can be activated into classically activated phenotype M1 or alternatively activated phenotype M2; the different phenotypes express a characteristic group of genes.

Figure 1

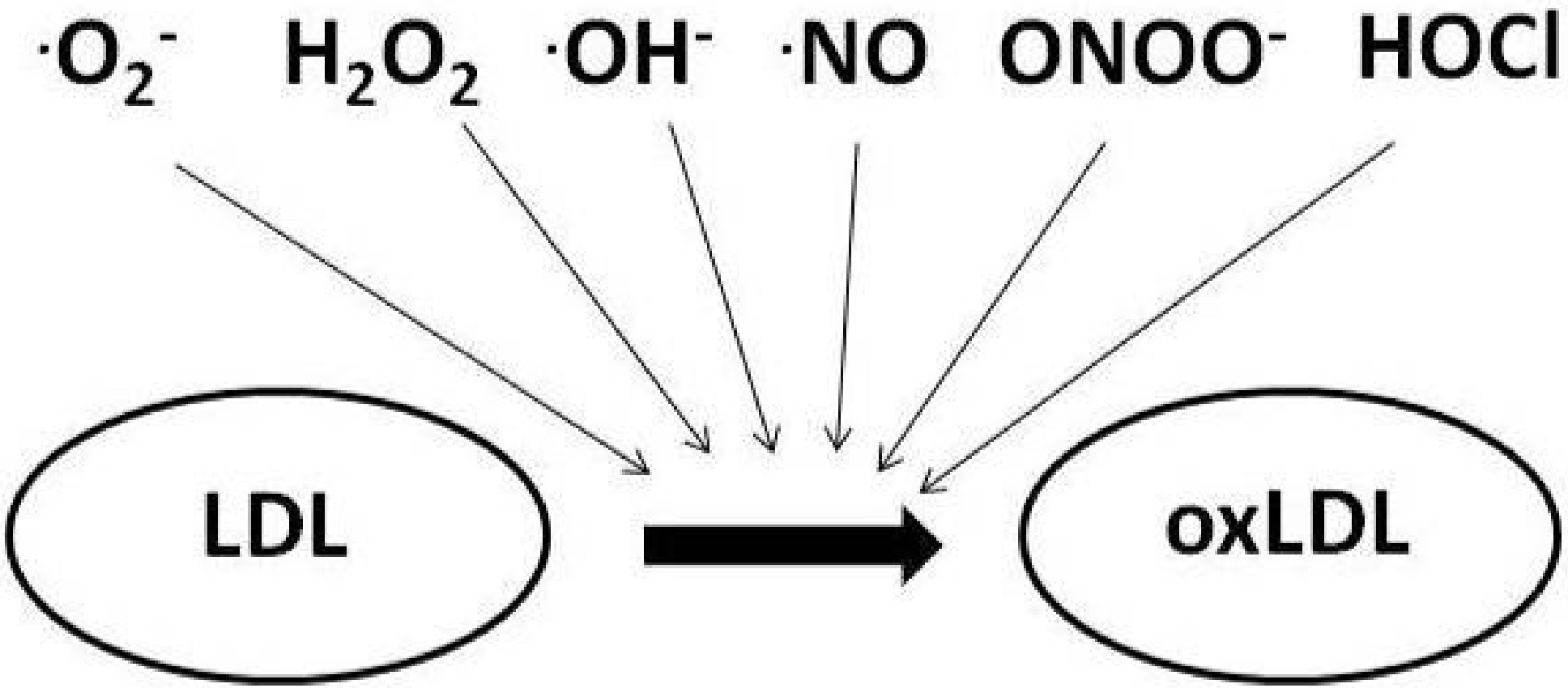

Figure 2

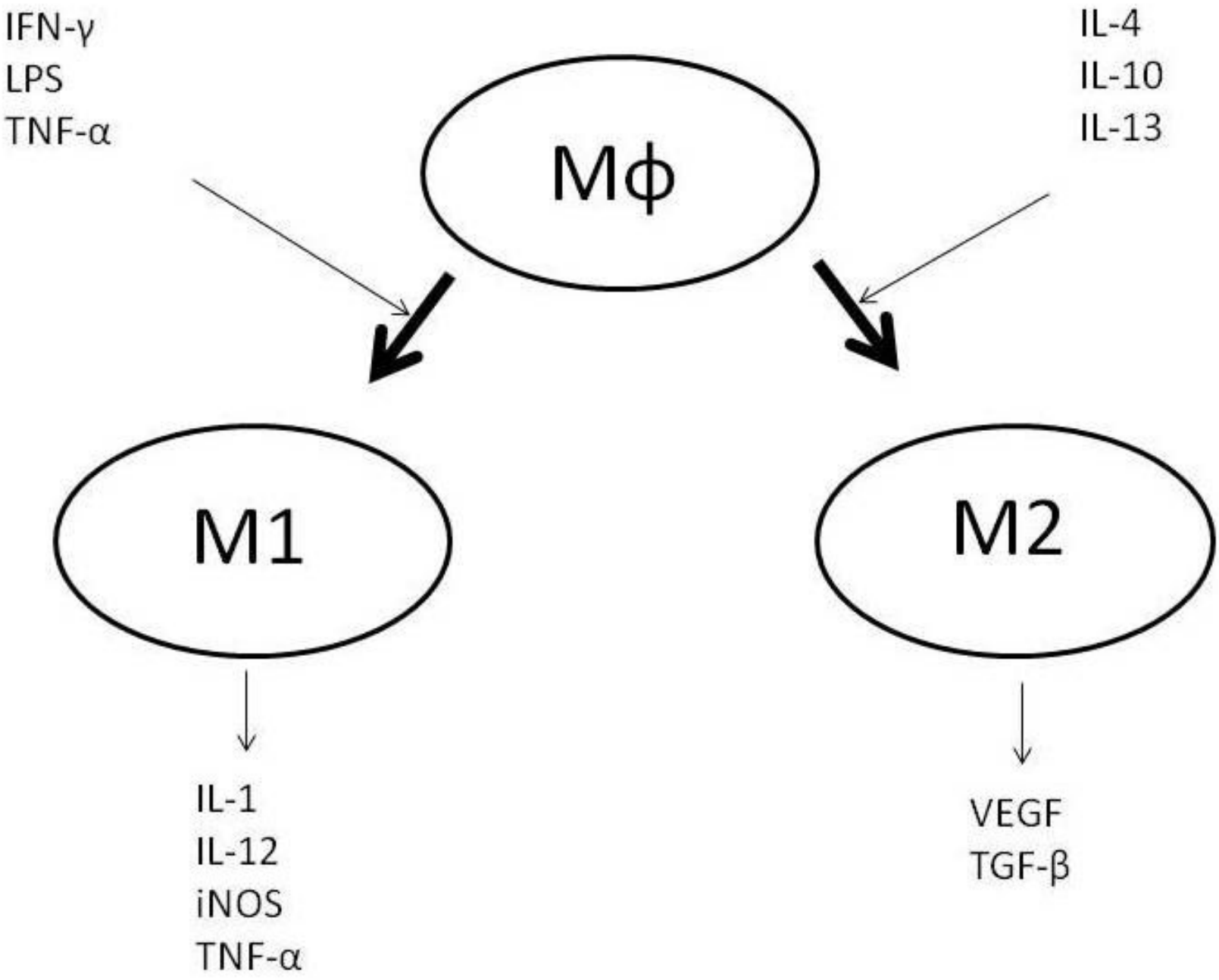
IFN-γ
LPS
TNF-α
Mϕ
IL-4
IL-10
IL-13
M1
M2
IL-1
IL-12
iNOS
TNF-α
VEGF
TGF-β

Table 1: Antioxidant therapy studies

Study	Sample	n	Findings	Reference
The Vitamin E Atherosclerosis Prevention Study (VEAPS) followed more than 300 patients for approximately 3 years receiving DL-α-tocopherol (400 IU per day) or placebo	Men and women ≥ 40 years old	300	Even with elevated plasmatic vitamin E and diminished circulating oxLDL, the treatment was not able to reduce the progression of intima-media thickness (IMT)	Hodis *et al*, 2002
RRR-α-tocopherol supplementation (1200 IU/day) for 2 years, in a randomized, controlled, double-bind trial	Men and women (40-70 years old) with coronary artery disease (CAD)	90	It was presented that the intervention increased plasmatic α-tocopherol and decreased plasmatic hsCRP and urinary F2-isoprostanes	Devaraj *et al*, 2007
The Los Angeles Atherosclerosis Study (cohort study) followed patients for approximately 18 months	Middle-aged (40-60 years old) men and women without symptomatic cardiovascular disease at baseline	573	After 18 months, carotid intima-media thickness was inversely related to 3 plasma oxygenated carotenoids (lutein, β-cryptoxanthin and zeaxanthin) and 1 plasma hydrocarbon carotenoid (α-carotene)	Dwyer *et al*, 2004
Patients received a single-dose (2g PO) and 500mg/day of vitamin C (in a randomized, double-blind, placebo controlled-study) for 30 days (long-term).	Patients (men and women) with angiographically documented CAD	46	Plasmatic vitamin C concentration and flow-mediated dilation (FMD) increased after single-dose and were sustained after long-term, while no differences were observed on placebo	Gocke *et al*, 1999
The *Endothelial Assessment of Risk from Lipids in Youth* (EARLY) Trial (a randomized, double-blind, placebo controlled-trial) followed children for 6 weeks under vitamins C (500 mg/day) and E (400 IU/day) supplementation	Boys and girls (9-20 years old) with familial hypercholesterolemia or the phenotype of familial combined hiperlipidemia	15	It was presented an improvement on endothelium-dependent FMD of the brachial artery. However, no differences were found regarding LDL oxidation, F2-isoprostanes, 8-hydroxy-2'-deoxyguanosine and hsCRP	Engler *et al*, 2003
The *Antioxidant Supplementation in Atherosclerosis Prevention* (ASAP) study evaluated the effect of supplementation with 91 mg d-α-tocopherol or 250 mg slow-release ascorbic acid or both for 6 years	Smoking and nonsmoking (men and postmenopausal women) aged 45 to 69 years old with serum cholesterol ≥ 5mmol/L	520	It was found a positive effect of vitamins C and E combination, since they could slow down the progression of atherosclerosis in hypercholesterolemic people	Salonen *et al*, 2003
A population-based study that followed subjects for seven years	Middle-aged (46-65 years old) men from eastern Finland	840	It was concluded that high serum levels of carotenoids can be protective against early atherosclerosis, since IMT was inversely correlated with lycopene, α-carotene and β-carotene	Karppi *et al*, 2013

Herein is presented a brief summary describing the main findings of selected studies that had humans as samples

CAPÍTULO II

Bioinformatics approach to evaluate differential gene expression of M1/M2 macrophage phenotypes and antioxidant genes in atherosclerosis

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Bioinformatics approach to evaluate differential gene expression of M1/M2 macrophage phenotypes and antioxidant genes in atherosclerosis

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Abstract

Atherosclerosis is a pro-inflammatory process intrinsically related to systemic redox impairments. Macrophages play a major role on disease development. The specific involvement of classically activated, M1 (pro-inflammatory), or the alternatively activated, M2 (anti-inflammatory), on plaque formation and disease progression are still not established. Thus, based on meta-data analysis of public micro-array datasets, we compared differential gene expression levels of the human antioxidant genes (HAG) and M1/M2 genes between early and advanced human atherosclerotic plaques, and among peripheric macrophages (with or without foam cells induction by oxidized low density lipoprotein, oxLDL) from healthy and atherosclerotic subjects. Two independent datasets, GSE28829 and GSE9874, were selected from Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/) repository. Functional interactions were obtained with STRING (http://string-db.org/) and Medusa (http://coot.embl.de/medusa/). Statistical analysis was performed with ViaComplex® (http://lief.if.ufrgs.br/pub/biosoftwares/viacomplex/) and Gene Score Enrichment Analysis (GSEA) (http://www.broadinstitute.org/gsea/index.jsp). Bootstrap analysis demonstrated that the activity (expression) of HAG and M1 gene sets were significantly increased in advanced compared to early atherosclerotic plaque. Increased expression of HAG, M1 and M2 gene sets were found in peripheric macrophages from atherosclerotic subjects compared to peripheric macrophages from healthy subjects, while only M1 gene set was increased in foam cells from atherosclerotic subjects compared to foam cells from healthy subjects. However, M1 gene set was decreased in foam cells from healthy subjects compared to peripheric macrophages from healthy subjects, while no differences were found in foam cells from atherosclerotic subjects compared to peripheric macrophages from atherosclerotic subjects. Our data suggest that, different to cancer, in atherosclerosis there is no M1 or M2 polarization of macrophages. Actually, M1 and M2 phenotype are equally induced, what is an important aspect to better understand the disease progression, and can help to develop new therapeutic approaches.

Key Words

Antioxidants. Atherosclerosis. Macrophages polarization. Bioinformatics. Gene expression.

1. Introduction

According to World Health Organization (WHO), about 30% of all deaths are attributed to cardiovascular diseases (http://www.who.int/en/). Atherosclerosis is one of the most important cardiovascular complications, which reflects an accumulation of lipids plaque on the media layer of the arterial wall. Even though it is difficult to identify primary events in long term process of atherosclerotic plaque formation, endothelial dysfunction, low density lipoproteins (LDL) oxidation and endocytosis of oxidized LDL (oxLDL) by infiltrated macrophages arewell established pathological factors in foam cells conversion, which are major triggers to atheroma plaque formation (1-5). Moreover, macrophages possess different classes of receptors that specifically recognize oxLDL (*e.g.:* CD36, SP-A), which, once stimulated, are responsible for the activation of a pro-inflammatory cascade, attracting more macrophages to the media layer. This inflammatory process has in the nuclear factor kappa B (NFκB) the major biological intermediate (6-9).

It is well known that free radicals and oxidants play an important role on disease progression. The role of myeloperoxidase (MPO)-derived hypochlorite (HOCl), nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-derived superoxide radicals ($^{\cdot}O_2^-$), transition metals and other oxidants have been largely studied (10). Besides its direct effects, superoxide can react with nitric oxide ($^{\cdot}NO$), generating peroxynitrite ($ONOO^-$), which can lead to protein modifications (11-13). Interestingly, reduced atherosclerosis burden was observed in carriers of hereditary deficiency of NADPH oxidase 2 (NOX2) (14). Moreover, elevated sulfhydryl (-SH)-protein oxidation is found in unstable plaques, mainly due to S-thiolation (15). However, antioxidants therapies present controversial and non satisfactory results (16).

Nowadays, the role of macrophages phenotype has been regarded in the atherosclerosis research. Activated macrophages can present two major phenotypes: the M1, or classically activated, which has pro-inflammatory features, and M2, or alternatively activated, with anti-inflammatory features (17, 18). Therefore, a predominance of M1 phenotype is observed in atherosclerosis (19-22). In cancer, there are evidences pointing to differential macrophages polarization during the disease progression, nevertheless with the conversion of an initial M1 into M2 predominance, representing a worst patient's prognosis by promoting tumor growth and metastasis (23, 24).

Bioinformatics tools are useful experimental approaches to help researchers to deal with systematic analysis of gene expression using high-throughput screening of cDNA microarray libraries. Several highly complex biological processes and pathological states have been uncovered with bioinformatics, such as cancer promotion and progression, diabetes complications, cardiovascular diseases and others (25-28).

Regarding all of these presented aspects, we were interested in establishing the potential role that M1/M2 phenotype modulation and human antioxidant genes have in atherosclerosis. Therefore, our aim in the present work was to compare gene expression levels between human advanced atherosclerosis plaques and human early atherosclerosis plaques, and among peripheric macrophages from atherosclerotic subjects, peripheric macrophages from healthy subjects, foam cells (induced, *in vitro*, by exposing macrophages to oxidized low density lipoprotein, oxLDL) from atherosclerotic subjects and foam cells from healthy subjects, considering three different gene sets: M1, M2 and human antioxidant genes (HAG).

2. Material and methods

2.1 Microarray datasets

First, we searched for datasets of interesting in Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/). Thus, we chose two different datasets: Gene Expression in Early and Advanced Atherosclerotic plaque from human carotid (GSE28829), and Expression Profiles of Human Macrophages (GSE9874).

2.1.1 GSE28829

Sixteen postmortem advanced (thin or thick fibrous cap atheroma) atherosclerotic plaques and thirteen postmortem early (intimal thickening and intimal xanthoma) atherosclerotic plaques, from human carotid artery, were retrieved from Maastricht Pathology Tissue Collection (MPTC)-Germany, being tissues obtained during autopsy (Department of Pathology, Maastricht University Medical Centre). Microarray expression

profile (mRNA) was determined by array with [HG-U133_Plus_2] Affymetrix® Human Genome U133 Plus 2.0 Array.

2.1.2 GSE9874

Microarray expression profiles (mRNA) of human peripheric macrophages were obtained from fifteen subjects with atherosclerosis/family history of coronary heart disease (CHD) and from fifteen subjects (sex and age matched) without atherosclerosis/family history of CHD. After collection, all monocyte-derived macrophages from peripheral blood were cultured in absence or presence (foam cells) of oxi-LDL from all subjects (healthy and atherosclerotic). Expression profile (mRNA) was determined by array with [HG-U133A] Affymetrix Human Genome U133A Array.

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2.2 Network Building

Different genes related with M1/M2 macrophage phenotype and human antioxidant genes (HAG) were selected. The M1 and M2 sets were built with an extensive literature review at PubMed http://www.ncbi.nlm.nih.gov/pubmed (29-31). Human Antioxidant Genes (HAG) set was built as previously described, (32). Additionally, HAG gene set was completed with nitric oxide-related genes, using two online platforms (with free access): String http://string-db.org/ and HUGO http://www.genenames.org/.

Finally, a gene network, containing M1/M2/HAG gene sets, was built with String platform. The prediction methods were neighborhood, gene fusion, co-occurrence, co-expression, experiments, databases and text mining, with a score of confidence = 0.400 (medium). Detailed description of the genes that compose the presented gene sets is available at Supplementary Material 1.

2.2 Gene Expression Network Analysis and Statistics

For differential gene expression analysis, we utilized the ViaComplex® software version 1.0 (Castro et al., 2009). The main advantage of this program is that it is able to distribute a given quantity (quantitative or qualitative data) onto gene/protein interaction networks. To do this, ViaComplex® overlaps functional

information (*e.g.* microarray data) with interaction information (supplied by the gene network built). We utilized statistical analysis available in the ViaComplex® package, which estimates the relative expression level of Groups of Functionally Associated Genes (GFAGs) and is describe elsewhere (33). Briefly, to obtain a quantitative parameter that characterizes the functional state of each Group of Functionally Associated Genes (GFAG) in the sample, ViaComplex® measures the information content using Shannon's entropy (34).

Separately, gene sets were analyzed by Gene Score Enrichment Analysis (GSEA), with $P \leq 0.05$ as significance level. Besides the expression data comparisons, GSEA offers a core of genes that contribute more for the score enrichment, presented by a heat map (35).

3. Results and Discussion

The present work investigated differential gene expression levels involved with antioxidant defense (the human antioxidant genes) and M1/M2 macrophage phenotypes in several aspects involved with atherosclerosis. The described disease has a complicated development mechanism, which justifies different efforts to better understand its pathophysiology. Herein, we evaluated two different datasets, first based on samples obtained from human atherosclerotic plaques (advanced *vs.* early) and second with microarray data from human macrophages from healthy and atherosclerotic subjects.

The resulting gene network presented in Figure 1, contain all M1, M2 and HAG gene sets evaluated in our study. More than just demonstrate the differences obtained in gene expression levels between groups, we also present a list of gene set enrichment in each comparison, which are summarized in Table 1. Gene expression comparisons among peripheric macrophages from atherosclerotic subjects, peripheric macrophages from healthy subjects, foam cells from atherosclerotic subjects and foam cells from healthy subjects, generated by ViaComplex, are at Figure 2. No differences were found about diversity in results presented at Figures 2A and 2B, indicating homogeneity in genes distribution, while activity (expression levels) differences are indicated at the figures. However, increase of diversity (homogeneity) was found for HAG at Figure 3A, while decrease was found for HAG at Figure 3B. Table 1 displays gene expression comparisons among peripheric

macrophages from atherosclerotic subjects, peripheric macrophages from healthy subjects, foam cells from atherosclerotic subjects and foam cells from healthy subjects, also generated by GSEA.

Analyzing the effect of disease on macrophages, differences were more prominent in peripheric macrophages than foam cell induction effects (performed in culture by treatment with oxLDL). We found elevated M1 genes, M2 genes and HAG sets expression in peripheric macrophages from atherosclerotic subjects compared to peripheric macrophages from healthy subjects, what is similar to plaque findings. There is a growing interesting in monocytes biomarkers for cardiovascular diseases, and the most important marker associated is CD16, since association of CD16+ monocytes with atherosclerosis is well-established. Additionally, other biomarkers have been associated with cardiovascular diseases, such as CD18, C11b, CXR1, CD36 and STAB1 (36, 37). However, only M1 gene set presented increased gene expression in foam cells from atherosclerotic subjects compared to foam cells from healthy subjects. Interestingly, with ViaComplex analysis, a diminished expression of M1 genes set was observed in foam cells from atherosclerotic subjects compared to peripheric macrophages from atherosclerotic subjects, and no differences between foam cells from healthy subjects and peripheric macrophages from healthy subjects. The above mentioned results were a little surprisingly, mainly in healthy subjects, since a pro-inflammatory profile (M1) would be more consistent with the induction of foam cell formation. Regarding redox system, GSEA analysis showed an increase in HAG set gene expression after foam cell induction. The last result leads us to propose a different effect of oxLDL on macrophage polarization, since different oxidants yield different modifications on this lipoprotein (22, 38, 39). So, can different oxidants generate different profiles on macrophage phenotype? More studies are needed to try to answer this question.

The comparison between gene expression levels of selected networks in advanced atherosclerotic plaque *vs*. early atherosclerotic plaque, analyzed by ViaComplex tool is shown in Figure 4. No differences were found related to samples diversity, indicating homogeneity in genes distribution (data not shown), while differences in activities (gene expression levels) are indicated in the figure. Besides it, Table 1 presents gene set enrichment analysis, which plays results in accordance to Viacomplex analysis.

When the datasets derived from advanced *vs*. early atherosclerotic plaques were compared, we observed an increased expression of M1 genes and HAG sets in advanced atherosclerotic plaques (Figure 4). Atherosclerosis is a pro-inflammatory disease and a polarization of M1 macrophage phenotype has been

identified in previous works (21, 40). However, even though a not significant increase in M2 dataset was found (P = 0.0672), what is consistent taken together with the significant increasing found by GSEA. Nevertheless, our results are in accordance to other previous works, which demonstrated that both M1 and M2 are enhanced in atherosclerosis (41, 42). It is well established the role of M1 phenotype in atherosclerosis, which is recognized as a pro-inflammatory process, regulated mainly by the expression of TNF-α, IL-1β, IL-6 and IL-12 (29, 41, 43). In contrast, the role played by M2 macrophages in atherosclerosis is still debated. Previous studies showed a decrease in M2 markers (e.g.: IL-4, IL-10 and IL-13), but most of them were performed in cell cultures (21, 40). Therefore, different roles are attributed to M2 phenotype in atherosclerosis. Some researchers correlate an increase in M2 phenotype with a protective role while others think that it is a part of the disease process, where M2 can express matrix metalloproteases, what in advanced atherosclerosis is an important factor to plaque disruption. Another different feature of M2 macrophages is their inability to phagocyte the oxLDL (44).

Moreover, HAG activity was also increased in advanced plaque. Despite the existence of many studies regarding the redox status of atherosclerosis, collectively they show conflicting and inconclusive results (40, 45). As previously showed, deletion of NADPH oxidase 1 (NOX1) results in an anti-atherosclerotic effect associated with a diminished ROS production in human aortic endothelial cells exposed to hyperglycemic conditions (45). High nitric oxide synthase (NOS) expression can lead to peroxynitrite formation, which ultimately leads to protein nitration. Additionally, the truncated form of thioredoxin 80 (Trx-80) is able to promote the macrophage differentiation to M1 phenotype (40). Thus, it is difficult understand if the antioxidants in advanced plaque are protective or attenuate the disease process.

Finally, GSEA analysis gives us a subset of genes (players) that more contributes to the observed enrichment. This approach drives us in further experiments, since a reliable phenotype signature is offered. Table 2 presents M1 and M2 players for advanced plaque and it is interesting observed MMP9 as a M1 phenotype player. Many reports have associated matrix metalloproteinase 9 enzyme (MMP-9) with atherosclerosis and cardiovascular risk (46-48). Additionally, CD14 and CD36 are presented as players for M2 phenotype, what is in accordance to previous works, which demonstrated these antigens as important mediators of inflammatory process (49-53). Recently, a cohort study with 951 patients concluded that CD14++CD16+ monocytes can independently predict cardiovascular events, being an interesting target for new therapies (54).

Table 3 presents the HAG up-regulated genes for foam-induced macrophages and advanced plaques. It was observed consistence between sets for the follow gene classes: nitric oxide synthases (NOS3, NOS1) and thiol redox (MT1G, MT1X, MT2A, MT1H, TXNDC5, GLRX, MT1F). The thiol redox can be an important target for study and therapy, since it was previously demonstrated there is a higher protein-SH oxidation in unstable plaques (55). However, the role of metallothioneins keeps still lacking for more studies relating to atherosclerosis. Hence, a study with diabetic and atherosclerotic old patients identified a novel 209/G MT2A polymorphism in this population (56). Curiously, our findings pointed to increased NOS1 and NOS3 in advanced plaque and in foam induced macrophage, what also suggests an important role of this enzyme, which is responsible for nitric oxide synthesis and can yields superoxide in stress situations. Indeed, formation of atherosclerotic plaque, inflammation process and interaction leukocyte-endothelium are associated with reduced superoxide production in apolipoproteinE/eNOS double knockout mice (57). Besides it, we found SOD-1 (cytosolic) in the signature of foam-induced macrophages, and SOD-2 (mitochondrial) in advanced plaque. Therefore, as discussed above, this enzyme has an important role in atherosclerosis, and our results corroborate by previous study that identified increased MnSOD (SOD-2) in atherosclerotic human aorta by immunohistochemical evaluation (58). Moreover, an interesting clinical trial demonstrated the efficacy of SOD. The GliSODin supplementation, a vegetal SOD that is associated with gliadin, was efficient in controlling the carotid thickness in adults aged 30-60 (59). Another interesting trial showed in 492 patients undergoing coronary artery bypass graft surgery that the drug atorvastatin diminishes vascular superoxide production and ameliorates nitric oxide bioavailability, and the mechanism is via tetrahydrobiopterin-mediated eNOS coupling (60).

4. Conclusion

We concluded that the role of M2 phenotype in plaques should be better explored, since not only M1 phenotype is present in the disease. Literature findings point to inflammatory response of M1 phenotype, but the way that we see M2 phenotype is not clear, mainly when considering *in vivo* models, which present considerable differences compared with *in vitro*. Additionally, the redox system is yet without more conclusive responses, mainly about mechanism and antioxidant therapies. However, our work was able to indicate some

specific candidates to more detailed validation, as SOD and NOS enzymes, which had expressive response of enrichment score. Thus, our findings could clearly impact cardiovascular complications and therapy. Ultimately, clinical relevance of our findings, as all promising biomarkers, requires prospective validation in carefully designed randomized, large-scale, clinical trials.

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Figure Captions

Figure 1: STRING 9.0 gene interactions network. Classically-activated (M1) macrophage phenotype gene set is presented in red circles. Alternative-activated (M2) macrophage phenotype gene set is presented in blue circles. Human antioxidant genes (HAG) set is presented in black circles.

Figure 2: Gene expression by ViaComplex®, using network from Figure A as a base. Human antioxidant genes (HAG) set is up and left. Macrophage pro-inflammatory phenotype (M1) genes set is up and right. Macrophage anti-inflammatory phenotype (M2) genes set is down. White hash means significant expression decreasing. Coordinates (X- and Y-axis) represent normalized values of the input network topology. Color gradient (Z-axis) represents the relative functional state mapped onto graph according to the data input, where $z = a/(a+b)$. The a is greater than b when $z>0.55$ (yellow to red), lower than b when $z<0.45$ (cyan to blue) and equivalent to b when $0.45<z<0.55$ (green). A. Comparison between peripheric macrophages from atherosclerotic subjects and peripheric macrophages from healthy subjects. Data input: a (peripheric macrophages from atherosclerotic subjects) vs. b (peripheric macrophages from healthy subjects). B. Comparison between foam cells from atherosclerotic subjects and foam cells from healthy subjects. Data input: a (foam cells from atherosclerotic subjects) vs. b (foam cells from healthy subjects).

Figure 3: Gene expression by ViaComplex®, using network from Figure A as a base. Human antioxidant genes (HAG) set is up and left. Macrophage pro-inflammatory phenotype (M1) genes set is up and right. Macrophage anti-inflammatory phenotype (M2) genes set is down. White hash means significant expression decreasing. Coordinates (X- and Y-axis) represent normalized values of the input network topology. Color gradient (Z-axis) represents the relative functional state mapped onto graph according to the data input, where $z = a/(a+b)$. The a is greater than b when $z>0.55$ (yellow to red), lower than b when $z<0.45$ (cyan to blue) and equivalent to b when $0.45<z<0.55$ (green). A. Comparison between foam cells from atherosclerotic subjects and peripheric macrophages from healthy subjects. Data input: a (foam cells from atherosclerotic subjects) vs. b (peripheric macrophages from healthy subjects). B. Comparison between foam cells from healthy subjects and peripheric macrophages from healthy subjects. Data input: a (foam cells from healthy subjects) vs. b (peripheric macrophages from healthy subjects).

Figure 4: Gene expression by ViaComplex®, using network from Figure A as a base. Human antioxidant genes (HAG) set is up and left. Macrophage pro-inflammatory phenotype (M1) genes set is up and right. Macrophage anti-inflammatory phenotype (M2) genes set is down. White hash means significant expression decreasing. Coordinates (X- and Y-axis) represent normalized values of the input network topology. Color gradient (Z-axis) represents the relative functional state mapped onto graph according to the data input, where $z = a/(a+b)$. The a is greater than b when $z>0.55$ (yellow to red), lower than b when $z<0.45$ (cyan to blue) and equivalent to b when $0.45<z<0.55$ (green). 1. Comparison between advanced atherosclerotic plaque and early atherosclerotic plaque. Data input: a (advanced atherosclerotic plaque) vs. b (early atherosclerotic plaque).

Figure 1

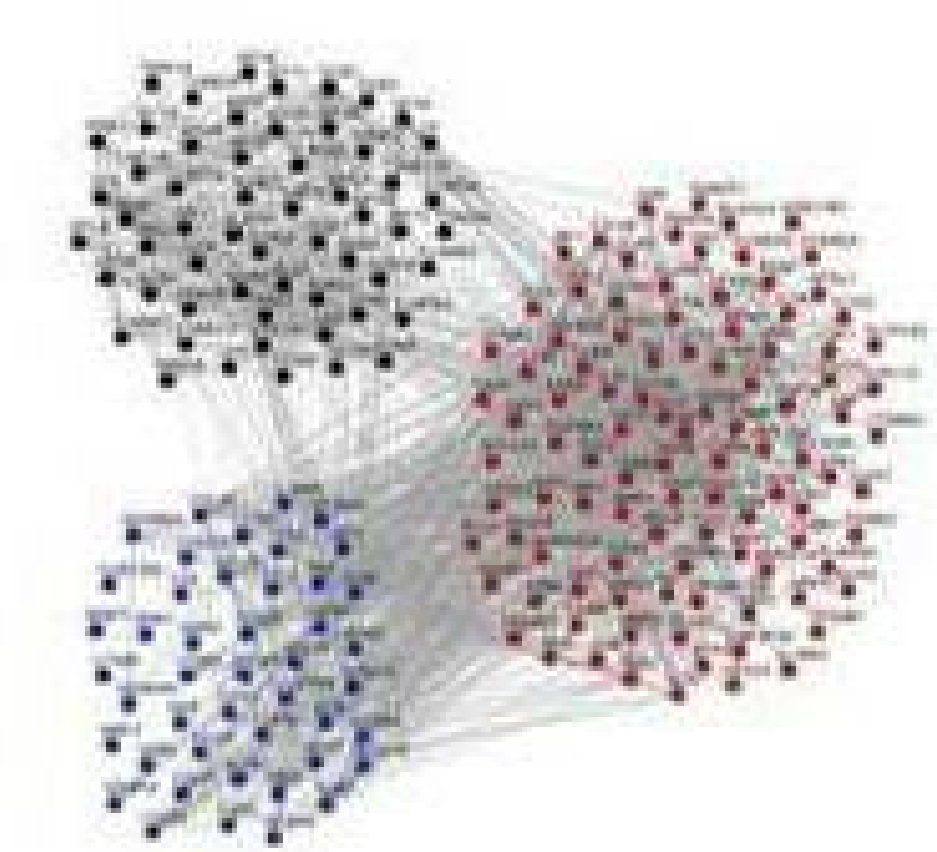

Figure 2

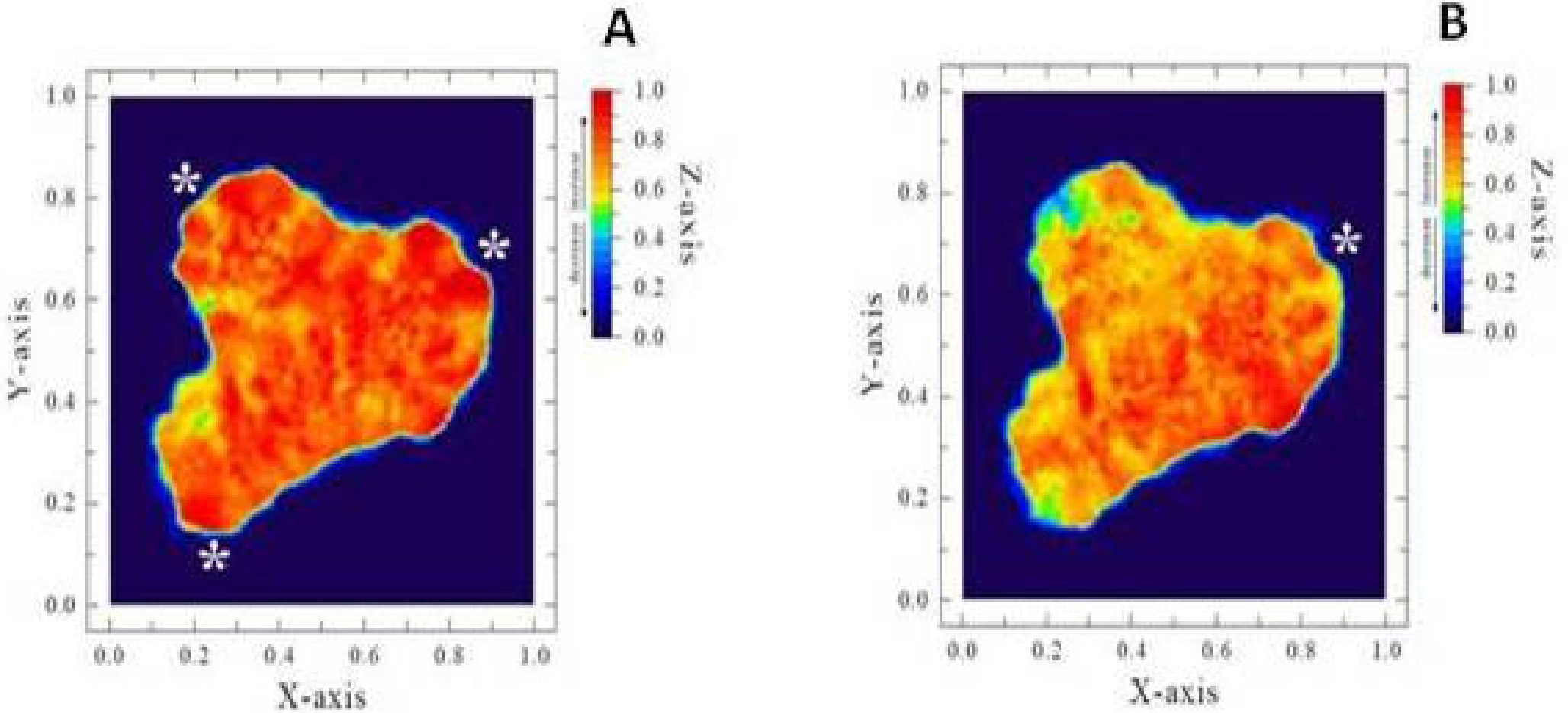

Figure 3

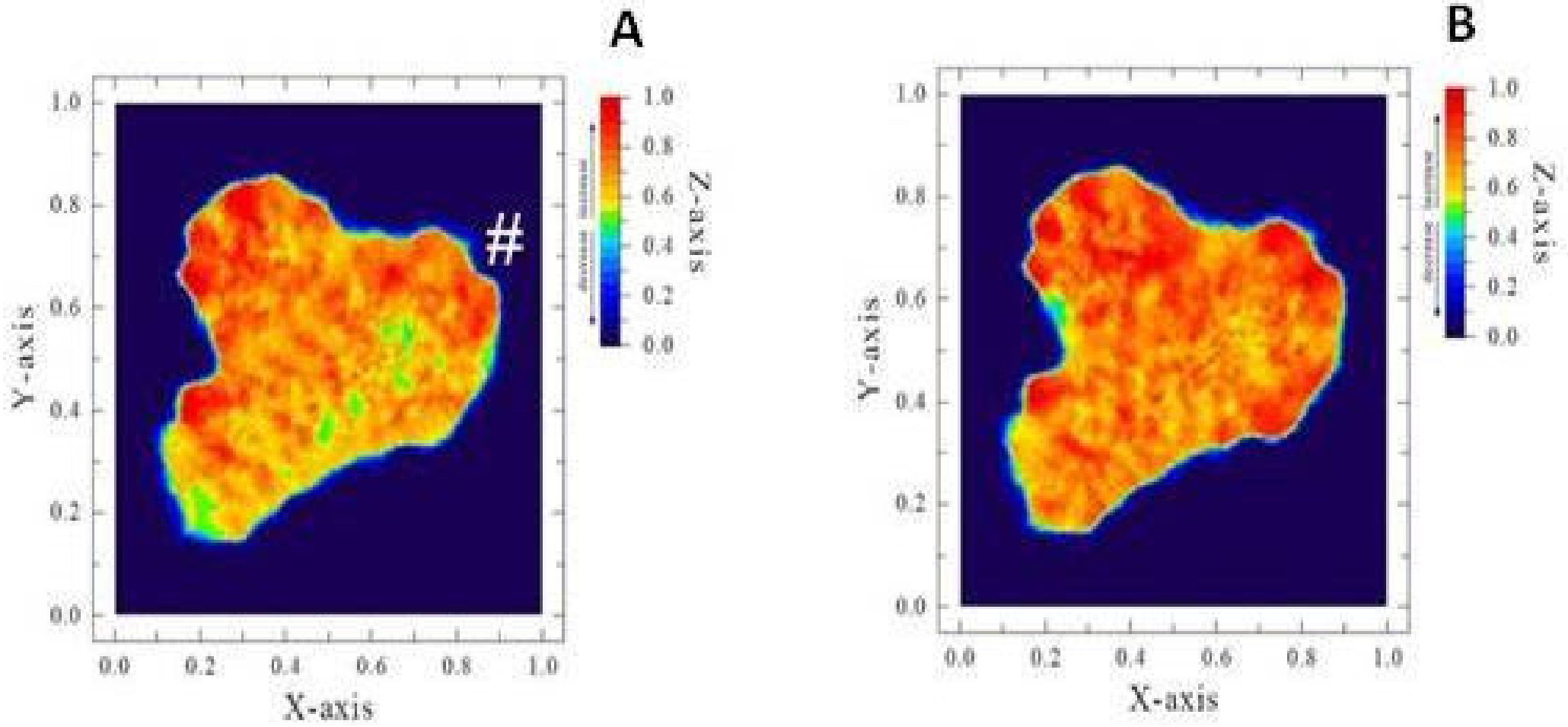

Figure 4

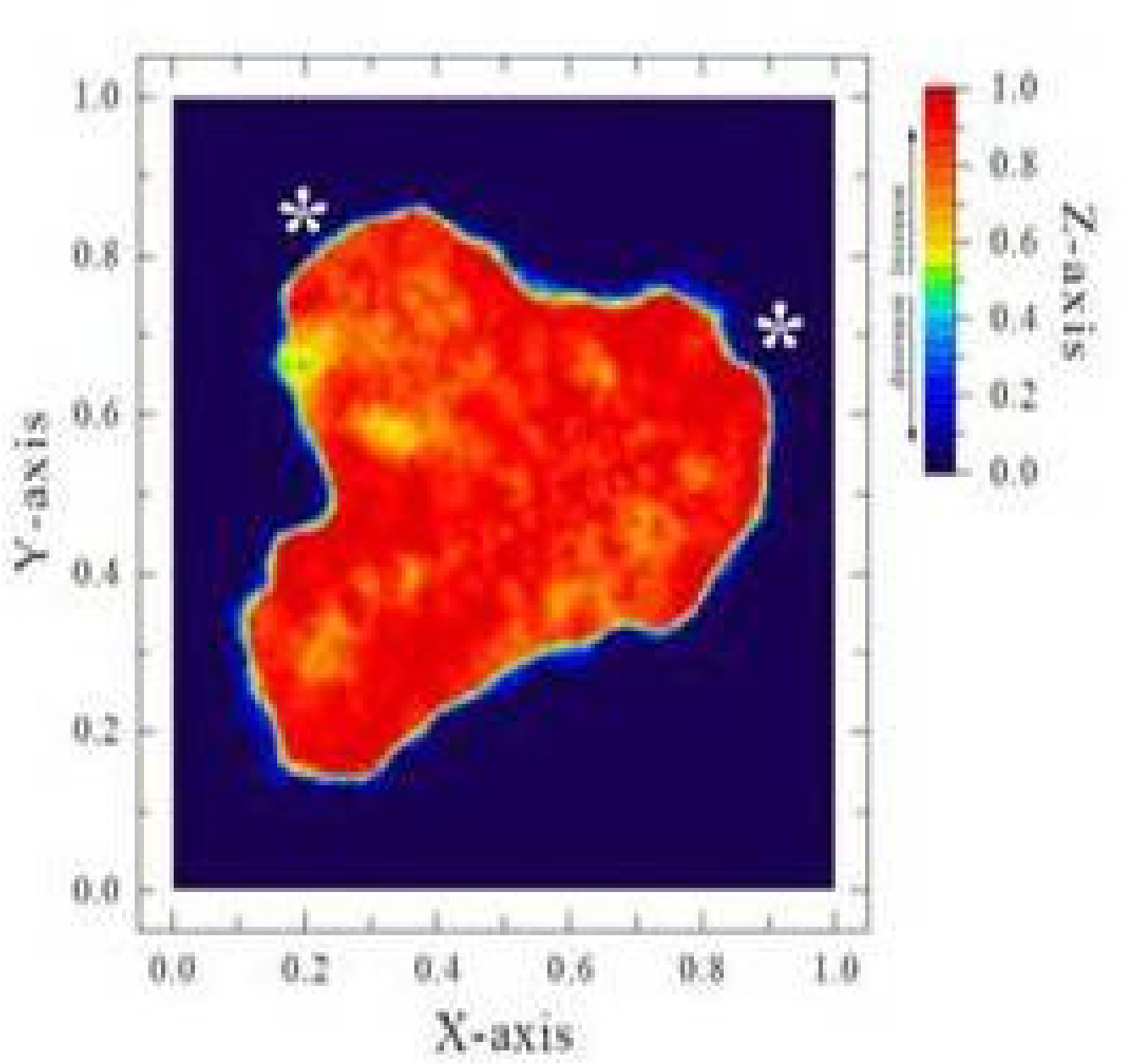

Table 1: Gene Set Enrichment Analysis

COMPARISONS	M1	M2	HAG
BA x BN	No difference	No difference	No difference
FA x FN	No difference	No difference	No difference
FN x BN	No difference	No difference	FN > BN
FA x BA	No difference	No difference	FA > BA
AP x EP	AP > EP	AP > EP	AP > EP

Comparisons considering the following sets: macrophage pro-inflammatory phenotype (M1) genes, macrophage anti-inflammatory phenotype (M2) genes and human antioxidant genes (HAG). ">" means gene set upregulated with nominal P value lower than 5%, "<" means gene set donwregulated with nominal P value lower than 5%, "No difference" means nominal P value higher than 5%. BA = Baseline macrophages from atherosclerotic subjects. BN = Baseline macrophages from healthy subjects. FA = Foam cells from atherosclerotic subjects. FN = Foam cells from healthy subjects. n = 15 for each one fo the groups BA, BN, FA, FN. AP = Advanced atherosclerostic plaque (n = 16). EP = Early atherosclerotic plaque (n = 13).

Table 2: Up-regulated genes for advanced atherosclerotic plaques

	ADVANCED PLAQUES *VS.* EARLY PLAQUES	
CLASSES	**M1**	**M2**
Enzymes	MMP9, MMP7, OAS2, PSMB9, SPHK1, TPSAB1, HSD11B1	CTSC, CA2, TPST2
Receptors	CD89, FCGR1A, FCGR2A, CSF1R, IL7R, TLR2, IL6R, CD80, IL15RA, ITGA2, IL1R1	MSR1, CXCR4, CD14, IL1ORA, CD36, CD163, TLR5, CCR2, SCARB1
Cytokines	IL8, EDN1, IL6, TNFSF10, IL15	IGFBP4, TGFBI, TGFBP1, IL10, IL1B, IGFBP3, IGF1, HRH1, TGFB1
T.F.	IRF1, IRF7	MAF, EGR2
Proteins	SPP1, CCL19, BCL2A1, LY96, MMRN1, CCL4, RGS1, SLC31A2, CCL2, CCL5, CCL20, PTX3, APOL6, IGFBP4, FAIM3, CXCL10, CXCL11, APOL1, APOL3, FADD	CCL18, MS4A4A, CLEC7A, SLCO2R1, MS4A6A, CCL13, CCL23, P2RY13, CCL16, CXCL12, CLEC1OA, CCL27, CCL22, GAS7, CLEC4M

GSEA analysis presenting a core of genes that more contribute to observed score enrichment modifications. T.F. = transcription factors.

Table 3: Up-regulated genes of Human Antioxidant Genes (HAG)

CLASSES	Foam cells from atherosclerotic *vs.* Peripheric macrophages from atherosclerotic	Advanced plaques vs Early plaques
Nitric oxide synthases	NOS3, NOS1	NOS3, NOS1
Peroxidases	CAT, PRDX2, GPX4	CP, GPX1, PRDX4, GPX7, GPX2, PRDX5, LPO
Superoxide dismutases	SOD1	SOD2
Thiol redox	TXNRD1, MT1G, TXN, TXNRD2, MT1X, MT2A, MT1H, TXNDC5, MSRA, GLRX, MT1F	GLRX, MT1G, TXNDC5, TXNDC12, TXN2, MT1E, MT1F, MSRA, MT1H, TXNDC3, MT2A, TXN, MT1X, TXNDC8, TXNL1

GSEA analysis presenting a core of genes that more contribute to observed score enrichment modifications.

PARTE III

DISCUSSÃO

A presente tese descreveu, em um primeiro momento, os dados presentes na literatura à cerca da ação de diferentes oxidantes na aterosclerose, bem como o papel da oxidação de LDL e dos diferentes tipos de fenótipos de macrófagos nessa doença. Em uma segunda etapa, foi feito um estudo comparativo de bioinformática (por biologia de sistemas), onde foram analisadas as expressões gênicas de três grandes grupos: genes antioxidantes humanos (HAG, do inglês *human antioxidant genes*), genes que caracterizam o fenótipo M1 e genes que caracterizam o fenótipo M2. Para tal análise, duas plataformas foram selecionadas: uma com dados de microarranjo de placas de aterosclerose humanas em estágio inicial e em estágio final e dados de microarranjo de macrófagos oriundos de pessoas ateroscleróticas ou saudáveis (com ou sem indução *in vitro* de formação de *foam cells*).

No capítulo 2 (parte 2) está apresentada a rede de expressão gênica com HAG, M1 e M2, apresentada como figura 1, enquanto as comparações (tanto de macrófagos quanto de placas) de enriquecimento de grupos de genes (GSEA, do inglês gene *set enrichment analysis*) estão apresentadas na tabela 1.

As comparações de expressão gênica, geradas por análise no ViaComplex, envolvendo os macrófagos de pessoas com aterosclerose, macrófagos de pessoas saudáveis, *foam cells* de pessoas com aterosclerose e *foam cells* de pessoas saudáveis são apresentadas nas figuras 2 e 3 (capítulo 2, parte 2). Primeiramente, analisando o efeito da doença, foi possível observar mudanças mais acentuadas nos macrófagos não estimulados do que nos *foam cells*; indução de formação de *foam cells* foi realizada *in vitro* por tratamento com oxLDL. Os grupos de genes HAG, M1 e M2 estão aumentados nos macrófagos de pacientes com aterosclerose em relação aos macrófagos de pessoas saudáveis. No entanto, somente o grupo M1 está aumentando em *foam cells* de pacientes com aterosclerose em comparação com *foam cells* de

pessoas saudáveis. Por outro lado, a análise do ViaComplex mostrou uma diminuição de M1 em foam cells de pessoas saudáveis em comparação com macrófagos não estimulados do mesmo grupo de indivíduos; para pessoas com aterosclerose, não há diferenças entre *foam cells* e macrófagos não estimulados. Pela análise do GSEA, HAG está aumentado em *foam cells* em ambos os grupos de indivíduos.

As comparações dos grupos de genes de interesse entre placas humanas em estágio avançado e placas humanas em estágio inicial, através de análise por ViaComplex, estão apresentadas na figura 4, enquanto as comparações feitas pelo GSEA estão na tabela 1. A lista de genes enriquecidos para os fenótipos M1 e M2 em placas em estágio avançado em comparação às placas em estágio inicial está apresentada na tabela 2; a tabela 3 apresenta a lista de genes enriquecidos de HAG em placas em estágio avançado em comparação com placas em estágio inicial (também são apresentados nessa tabela os genes enriquecidos de HAG em *foam cells* de pessoas ateroscleróticas comparados com macrófagos não estimulados desse mesmo grupo de indivíduos). Os resultados apontaram um aumento de M1 e de HAG em placas ateroscleróticas em estágio avançado.

Diferentes marcadores podem ser associados com doenças cardiovasculares, tais como CD16, CD18 e CD36. O CD16 tem sido relacionado como um dos principais marcadores, já que está bem associada uma relação entre monócitos $CD16^{+}$ com aterosclerose (Gratchev et al.; Gratchev et al.). A aterosclerose é uma doença pró-inflamatória e uma polarização do fenótipo M1 foi apresentada em alguns modelos. Entretanto, um aumento, mesmo que não significante ($P = 0,0672$), da expressão de genes do fenótipo M2 encontrada pelo ViaComplex está em acordo com a análise obtida pelo GSEA. Esses resultados vão ao encontro de trabalhos que encontraram ambos os fenótipos, M1 e M2, aumentados em aterosclerose (Devaraj and Jialal, 2011; Mahmood et al.).

O papel pró-inflamatório do fenótipo M1 já é bem entendido em aterosclerose, sendo regulado, principalmente, pela expressão de genes como TNF-α, IL-1β, IL-6 e IL-12 (Martinez et al., 2006; Martinez et al., 2008; Stoger et al.). Por outro lado, o papel do fenótipo M2 ainda está em grande discussão. Alguns trabalhos, realizados em cultura de células, encontraram diminuição, ao invés de aumento, em marcadores do fenótipo M2, como IL-4, IL-10 e IL-13 (Devaraj and Jialal, 2011; Mahmood et al.). A função do fenótipo M2 em aterosclerose tem recebido algumas hipóteses. Alguns pesquisadores entendem que seria uma tentativa de defesa do organismo, sendo sugerida, até mesmo, sua incapacidade de fagocitar oxLDL. Além disso, outros pesquisadores entendem que a função do fenótipo M2 é importante no desenvolvimento da doença devido à sua capacidade de expressar MMP (Hirose et al., 2011). Por outro lado, Cardilo-Reis e colaboradores demonstraram que o fenótipo M2 é capaz de lidar melhor com os lipídios do que o fenótipo M1 (induzido por IFN-γ) (Cardilo-Reis et al., 2012). Outro achado importante, nesse sentido, foi um estudo em que macrófagos oriundos de pacientes diabéticos foram tratados com oxLDL após parte deles terem sido estimulados ao fenótipo M1 e os outros terem sido estimulados ao fenótipo M2. A indução de receptores CD36 e SR-A1 resultou em uma maior formação de foam cells pelo estímulo de M2, em um mecanismo mediado por estresse de retículo endoplasmático (Oh et al., 2012). Uma interessante hipótese foi proposta por Khallou-Laschet e colaboradores, após suas observações, em modelo animal, de que lesões em estágio inicial apresentam maior proporção de fenótipo M1 (contribuindo para a proliferação de VSMC) e que, após isso, haveria uma troca na proporção (Khallou-Laschet et al., 2010).

Interessantemente, a tabela 2 apresenta o gene de MMP-9 como um dos genes característicos do fenótipo M1. A MMP-9 é uma metaloproteinase que tem sido associada à aterosclerose e com risco cardiovascular (Blin et al.; Pollanen et al., 2005; Ye, 2006). Nosso trabalho apresentou os genes de CD14 e CD36 para o fenótipo M2, o que vai ao encontro de

trabalhos prévios que apresentaram esses antígenos como mediadores do processo inflamatório (Hermann et al.; Konii et al.; Poitou et al.; Pu et al.; Zhou et al.). Recentemente, um estudo de corte com 951 pacientes concluiu que monócitos CD14+ e CD16+ podem, de forma independente, predizer eventos cardiovasculares, o que os torna alvos interessantes em termos de terapia (Rogacev et al.).

A indução do fenótipo M1 pode ser estimulada, *in vitro* e *in vivo* (modelo de ratos), pela proteína reativa C de alta sensibilidade, hsCRP (do inglês *high sensitivy C reactive protein*), uma proteína clinicamente usada como parâmetro para risco cardiovascular. Adicionalmente, é demonstrada também a capacidade da hsCRP em converter um fenótipo M2 (monócitos estimulados primariamente com IL-4) em M1 (Devaraj and Jialal, 2011). Complementarmente, Yamamoto e colaboradores sugerem que a angiotensina II exerce seus efeitos pró-aterogênicos por induzir o fenótipo M1 (Yamamoto et al., 2011).

Apesar de haver muitos estudos considerando os mecanismos de oxidação em aterosclerose, quando analisados em conjunto, dados conflitantes e inconclusivos são apresentados. Já foi demonstrado que a deleção do gene de NOX1 gera uma diminuição na produção de ROS em células endoteliais aórticas humanas em condições hiperglicêmicas (Gray et al.; Mahmood et al.). Um aumento na expressão de NOS, a qual poderia ter um efeito fisiológico positivo através de uma melhora na produção de óxido nítrico, leva à formação de peroxinitrito, que por sua vez induz a nitração de proteínas. Além disso, a forma truncada da Trx-80 pode promover a diferenciação de macrófagos no fenótipo M1 (Mahmood et al.). A tabela 3 do nosso estudo mostra uma consistência de genes HAG mais expressos entre placas em estágio avançado e *foam cells*; entre eles, veem-se o subgrupo das NOS (NOS3 e NOS1) e das enzimas tiol-redox (MT1G, MT1X, MT2A, MT1H, TXNDC5,GLRX, MT1F). O sistema tiol-redox tem aparecido como um interessante alvo, tendo, inclusive, sido demonstrada uma alta oxidação de proteínas contendo –SH em placas instáveis (Lepedda et al.). Por outro lado,

o estudo das metalotioneínas ainda carece de maior aprofundamento. Uma pesquisa com pacientes diabéticos e ateroscleróticos idosos encontrou um novo polimorfismo, o polimorfismo 209/G MT2A (Giacconi et al., 2005). Em ratos com duplo *knockout* para apoliproteína E e para eNOS, foi encontrada uma associação entre a produção de superóxido com formação de placa de ateroma, processo inflamatório e interação leucócito-endotélio (Ponnuswamy et al., 2012). Esse trabalho vai ao encontro dos nossos resultados para NOS3 e NOS1, bem como para SOD1 em *foam cells* e SOD2 em placas avançadas. Outro trabalho, com avaliação imunohistoquímica, identificou aumento de SOD2 em aorta aterosclerótica humana (Perrotta et al.). A realização de um ensaio clínico mostrou que a suplementação com GliSODin (SOD vegetal associada com gliadina) teve efeito no controle da espessura da carótida em adultos de 30 a 60 anos (Cloarec et al., 2007). Outro ensaio, com 492 pacientes submetidos à cirurgia arterial coronariana, demonstrou que a droga artosvastatina diminui a produção de superóxido e melhora a biodisponibilidade de óxido nítrico através do acoplamento da eNOS (Antoniades et al., 2011). A molécula antioxidante Trx apresenta efeitos sobre a diferenciação de macrófagos por promover o fenótipo M2 (induzido por IL-4 e IL-13) e por reduzir o fenótipo M1 (induzido por LPS). Efeitos semelhantes são encontrados em ratos hipolipoproteinêmicos ApoE2.Ki, os quais apresentam severos danos ateroscleróticos e uma maior população de M1 em relação a M2 quando desafiados com LPS. Por outro lado, a Trx é capaz de reverter uma predominância da população de fenótipos, bem como diminuir a área da lesão (El Hadri et al., 2012).

Hirose e colaboradores realizaram um estudo em que foi avaliada a expressão gênica em macrófagos não polarizados (M0), macrófagos M1 e macrófagos M2 após tratamento com oxLDL. Eles observaram, então, que a oxLDL interfere somente na função dos macrófagos M1 através da modulação dos genes de TNF-α e de NFκB (Hirose et al., 2011). Adicionalmente, alguns fenótipos alternativos de macrófagos também são sugeridos.

Macrófagos tratados com fosfolipídeos oxidados adquirem o fenótipo Mox, o qual é diferente de M1 e M2, apresentando baixa capacidade fagocítica e uma alta capacidade quimioatrativa. Além disso, o fenótipo Mox apresenta a expressão de genes regulatórios do sistema redox (os quais tem sua transcrição mediada por NrF2) como sendo característicos. No estudo de Kadl e colaboradores, os macrófagos Mox representaram 30% da população total de macrófagos presentes na área de lesão aterosclerótica de ratos $LDLR^{-/-}$ (Kadl et al., 2010). Um desconhecido fenótipo ateroprotetivo de macrófagos é estimulado pelo grupo heme, expressando fator de transcrição de ativação 1 (ATF-1) e sendo chamado de Mhem (Boyle et al., 2012).

Como a oxLDL está intimamente relacionada com a função dos macrófagos no desenvolvimento da aterosclerose, os mecanismos que levam à sua oxidação são de fundamental importância para que se possa melhor compreender a possível relação o tipo de oxidação e os diferentes fenótipos dessas células. A LDL pode ser oxidada por óxido nítrico devido à capacidade desse radical em atravessar superfícies, podendo então atingir o núcleo lipídico hidrofóbico (Denicola et al., 2002). LDL isolada da íntima de aorta humana aterosclerótica apresenta altos níveis de 3-nitrotirosina, sendo que a nitração proteica pode contribuir para o desdobramento da apolipoproteína B-100, apo B-100 (Hamilton et al., 2008; Leeuwenburgh et al., 1997). A nitração e a lipoperoxidação de LDL por RNS pode ser catalisada pela mieloperoxidase. Incubação de LDL com mieloperoxidase, nitrito e peróxido de hidrogênio gera nitração de resíduos tirosil de apo B-100 e início de lipoperoxidação. Essa LDL modificada induz a formação de *foam cells* (Podrez et al., 1999). Interessantemente, o dano induzido por peroxinitrito em LDL é inibido por metalotioneína (Cai et al., 2000).

Outra espécie envolvida com a oxidação de LDL é o hipoclorito, o qual oxida pontos da apo B-100. No entanto, outros pesquisadores sugerem que a reação do hipoclorito com a apo B-100 gera radicais secundários, os quais então seriam responsáveis pelo início da cascata

de lipoperoxidação. Opostamente, a vitamina C apresenta papel protetor ao dano induzido por hipoclorito (Carr et al., 2000; Hazell et al., 1999). Considerando ainda o efeito da mieloperoxidase, um estudo observou que a incubação de LDL com mieloperoxidase, peróxido de hidrogênio e íon cloro, em pH ácido, resultou na formação de esteróis clorados, sugerindo então o papel do ácido hipocloroso (ver equação 1). Porém, em um ambiente livre de íons cloro, o ácido hipocloroso não é capaz de gerar os mesmos esteróis clorados, sugerindo então o papel do cloro molecular (ver equação 2).

$$Cl^- + H_2O_2 + H^+ \longrightarrow HOCl + H_2O \text{ (Eq. 1)}$$

$$HOCl + H^+ + Cl^- \longrightarrow Cl_2 + H_2O \text{ (Eq. 2)}$$

Apesar do expressivo número de estudos com tratamentos antioxidantes em aterosclerose, os dados obtidos são bastante conflitantes. Shimada e colaboradores demonstraram que o antioxidante N-acetilcisteína pode reduzir a área da lesão aterosclerótica e a produção de radical superóxido em um modelo de ratos deficientes em apolipoproteína E (apo E), mas sem mudanças no perfil lipídico (Shimada et al., 2009). O estudo *Vitamin E Atherosclerosis Prevention Study* (VEAPS) acompanhou mais de 300 pacientes que receberam DL-α tocoferol ou placebo por aproximadamente 3 anos (400 $IU.dia^{-1}$). Mesmo que eles tenham apresentado níveis elevados de vitamina E plasmática e níveis diminuídos de oxLDL circulante, o tratamento não foi eficaz em reduzir a espessura da íntima-média (Hodis et al., 2002). Por outro lado, a suplementação de RRR-α-tocoferol por 2 anos (400 $IU.dia^{-1}$) aumentou os níveis plasmáticos de α-tocoferol e reduziu os níveis de hsCRP plasmática e de F2-isoporstanos urinários (Devaraj et al., 2007). O *Los Angels Atherosclerosis Study* (estudo de corte com 513 pacientes de meia idade) sugeriu que elevados níveis de carotenoides oxidados e α-caroteno podem apresentar um papel protetor em aterosclerose em estágio inicial (Dwyer et al., 2004). O ensaio clínico *Endothelial Assessment of Risk from Lipids in Youth* (EARLY) apresentou uma melhora na dilatação da artéria braquial de crianças com

hipercolesterolemia familiar após 6 semanas de suplementação com vitaminas C e E, mas não foram observadas mudanças quanto à oxidação de LDL, níveis de F2-isoprostanos, níveis de 8-hidroxi-2'-deoxiguanosina e níveis de hsCRP (Engler et al., 2003).

CONCLUSÃO

Concluímos com a presente tese que o papel do fenótipo M2 em aterosclerose deve ser melhorado investigado, já que não somente o fenótipo pró-inflamatório M1 está presente nessa doença e que diferenças consideráveis são encontradas entre modelos *in vitro* e *in vivo*. Além disso, apesar de haver uma grande quantidade de estudos a respeito do sistema redox, ainda há muitas dúvidas em relação à descrição de mecanismos específicos e, principalmente, há dados muito conflitantes em relação às terapias antioxidantes. Nesse sentido, nosso trabalho foi eficaz em apontar alvos para validação futura, como as enzimas SOD, NOS e tiol-redox (principalmente em relação às metalotioneínas, ainda pouco exploradas). Por fim, os mecanismos de oxidação de LDL podem representar um meio de comunicação entre os processos redox e a diferenciação fenotípica.

PERSPECTIVAS

Considerando que vários oxidantes estão envolvidos com o desenvolvimento da aterosclerose (podendo a LDL ser oxidada por vários deles através de diferentes formas), que, contrariamente, terapias antioxidantes apresentam resultados diversos e conflitantes e que os papéis dos fenótipos M1 e M2 precisam ser melhores compreendidos, entendemos que um passo seguinte seria a realização de um trabalho (ou mais de um, na verdade) relacionando esses pontos. Nesse sentido, as seguintes etapas futuras podem ser delineadas:

- estudo de bioinformática estrutural para simular a reatividade da LDL com diferentes alvos;

- estudo *in vitro* para descrever as modificações causadas por diferentes oxidantes na LDL (análises de oxi- e nitro-proteomas seriam bastante úteis entre as diversas análises que podem ser elaboradas);

- estudo com cultivo celular para tratar macrófagos com moléculas de LDL oxidadas por diferentes oxidantes, a fim de observar a possível indução de diferentes fenótipos.

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