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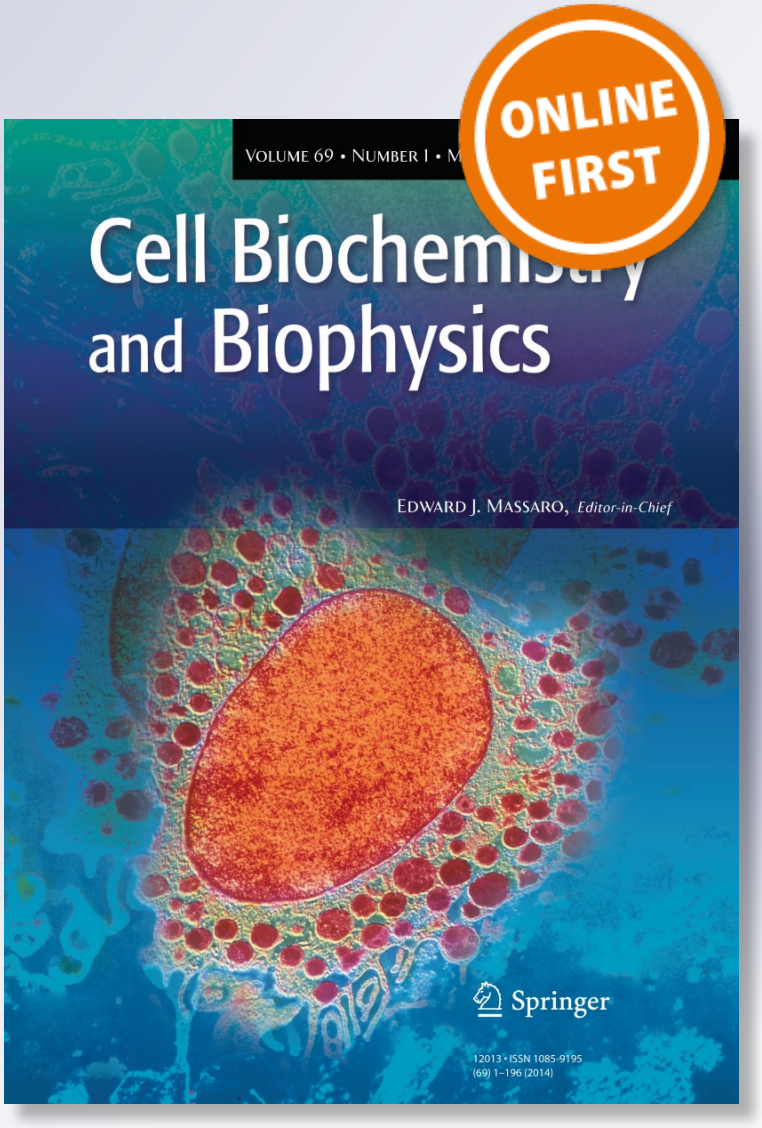

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ORIGINAL PAPER

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 (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 (http://www.broadinstitute.org/gsea/index.jsp). Bootstrap analysis demonstrated that the activity (expression) of HAG and M1 gene sets were significantly increased in advance compared to early atherosclerotic plaque. Increased expressions 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.

Keywords Antioxidants · Atherosclerosis · Bioinformatics · Gene expression · Macrophages polarization

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

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lipoproteins (LDL) oxidation, and endocytosis of oxidized LDL (oxLDL) by infiltrated macrophages are well-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 roles of myeloperoxidase (MPO)-derived hypochlorite (HOCl), nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-derived superoxide radicals ($^{\cdot}O2^{-}$), 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 (HGA) 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).

Materials and Methods

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).

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.

Gse9874

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.

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]. 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.

Gene Expression Network Analysis and Statistics

For differential gene expression analysis, we utilized the ViaComplex® software version 1.0 [27]. 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 described elsewhere [33]. Briefly, to obtain a quantitative parameter that characterizes the functional state of each 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].

Results and Discussion

The present work investigated differential gene expression levels involved with antioxidant defense HAG 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 Fig. 1 , contain all M1, M2, and HAG gene sets evaluated in our study.

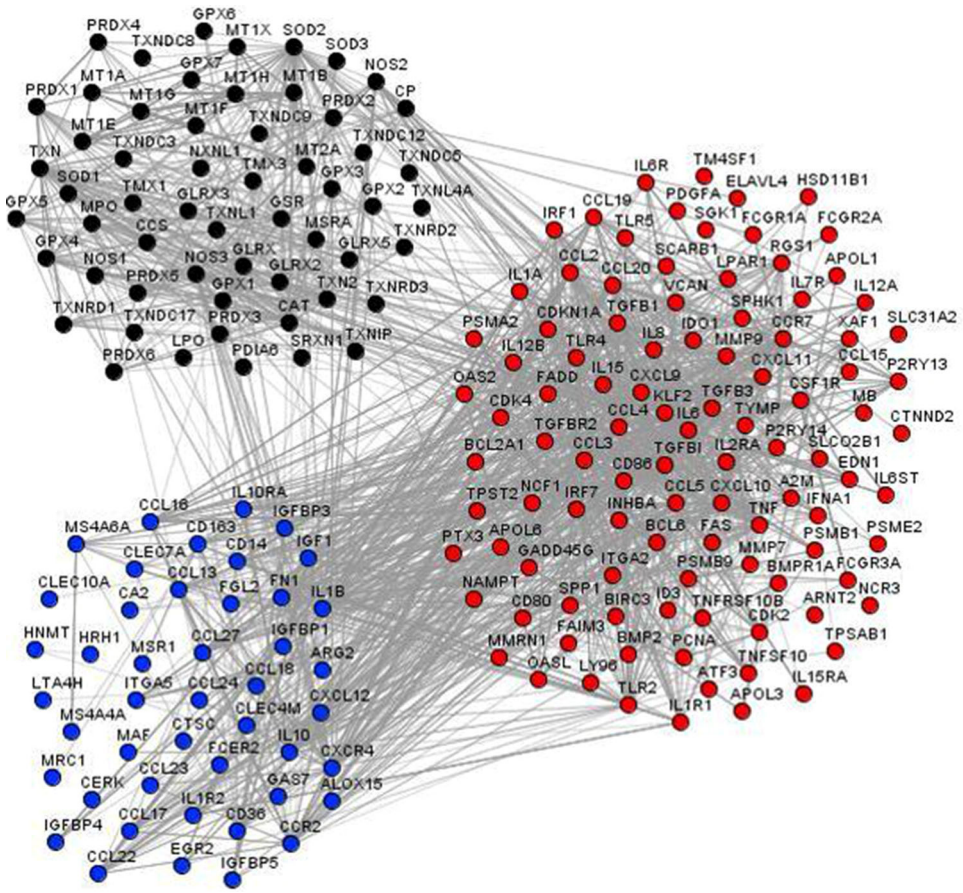

Fig. 1 STRING 9.0 gene interactions network. Classically activated (M1) macrophage phenotype gene set is presented in red circles. Alternative-activated (M2) macrophage phenotype gene set is presented in *blue circles*. Human antioxidant genes (HAG) set is presented in *black circles* (Color figure online)

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 Figs. 2 and 3. No differences were found about diversity in results presented at Fig. 2a, b, 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 Fig. 3a, while decrease was found for HAG at Fig. 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, which 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 STAB 1 [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.

Table 1 Gene Set Enrichment Analysis

Comparisons	Ml	M2	HAG
BA × BN	No difference	No difference	No difference
FA × FN	No difference	No difference	No difference
FN × BN	No difference	No difference	FN > BN
FA × BA	No difference	No difference	FA > BA
AP × EP	AP > EP	AP > EP	AP > EP

Comparisons considering the following sets: macrophage pro-inflammatory phenotype (Ml) 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 ($n = 13$)

The comparison between gene expression levels of selected networks in advanced atherosclerotic plaque and early atherosclerotic plaque, analyzed by ViaComplex tool, is shown in Fig. 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 2 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 (Fig. 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

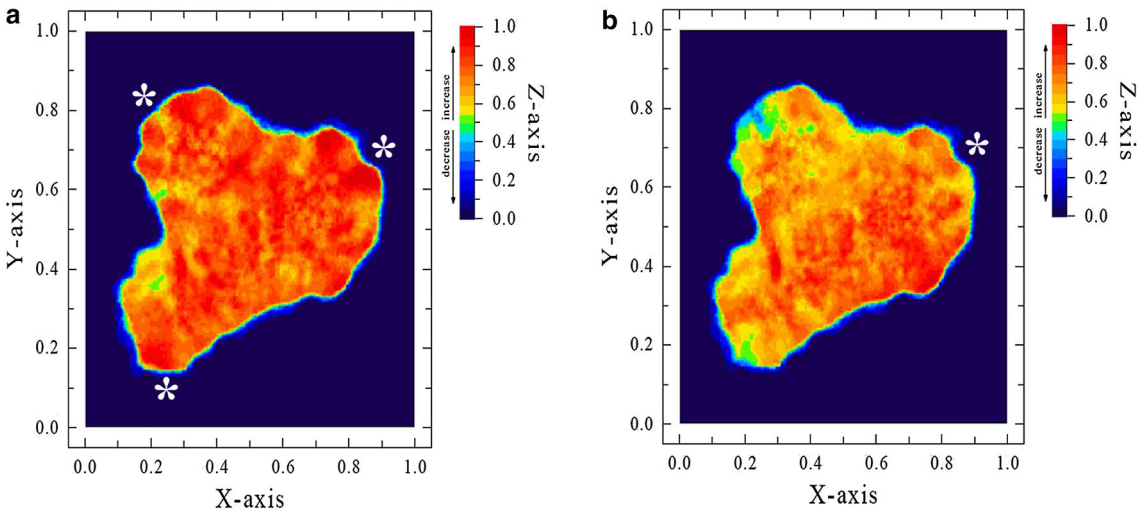

Fig. 2 Gene expression by ViaComplex®, using network from Fig. 1 as base. Human antioxidant genes (HAG) set is up and left. Macrophage pro-inflammatory phenotype (M1) genes set is up and right. Macrophage anti-inflammatory phenotype (M2) genes set is down. White hash means significant expression decreasing. 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) (Color figure online)

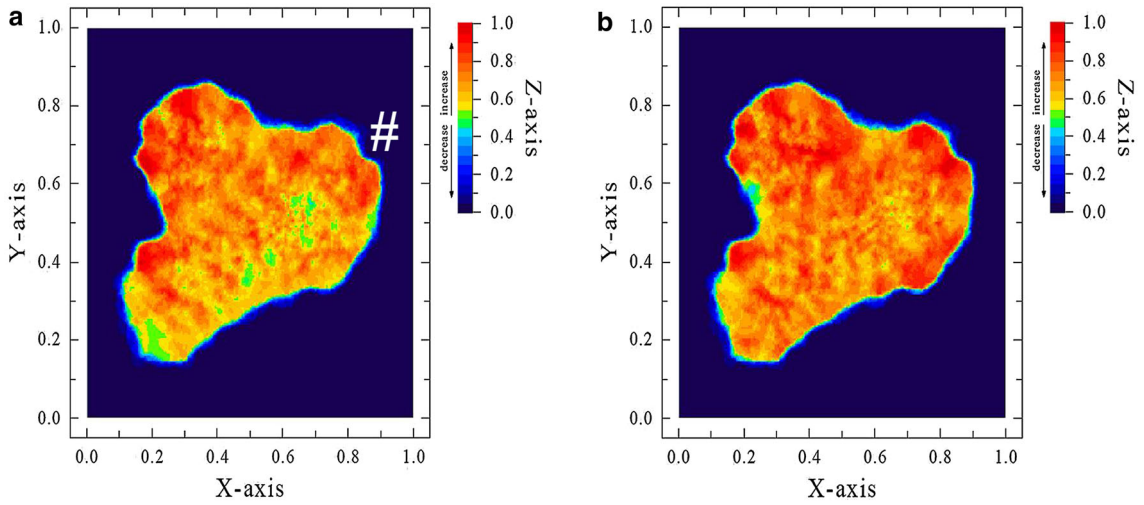

Fig. 3 Gene expression by ViaComplex®, using network from Fig. 1 as base. Human antioxidant genes (HAG) set is up and left. Macrophage pro-inflammatory phenotype (M1) genes set is up and right. Macrophage anti-inflammatory phenotype (M2) genes set is down. White hash means significant expression decreasing. 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) (Color figure online)

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

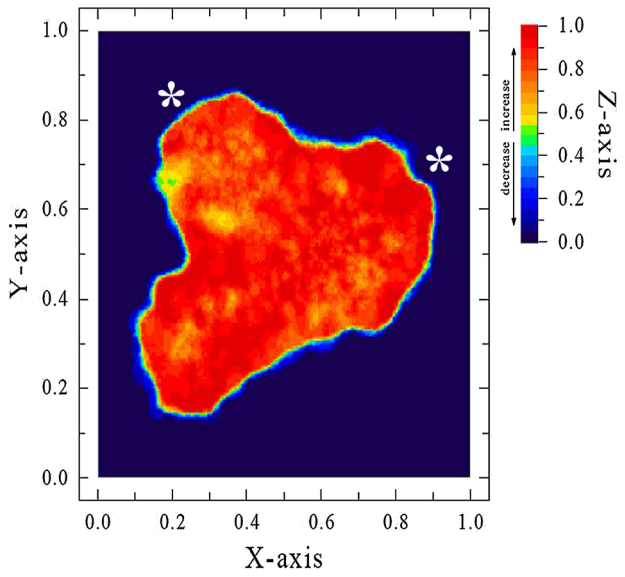

Fig. 4 Gene expression by ViaComplex®, using network from Fig. 1 as a base. Human antioxidant genes (HAG) set is up and left. Macrophage pro-inflammatory phenotype (M1) genes set is up and right. Macrophage anti-inflammatory phenotype (M2) genes set is down. White hash means significant expression decreasing. 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) (Color figure online)

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 to 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, interestingly, it is 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, which 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

Table 2 Up-regulated genes for advanced atherosclerotic plaques

Classes	Advanced plaques vs. early plaques	
	MI	M2
Enzymes	MMP9, MMP7, OAS2, PSMB9, SPHKLTPSAB1, HSD11B1	CTSC, CA2, TPST2
Receptors	CD89, FCGR1 A, FCGR2A, CSF1R, IL7R, TLR2, IL6R, CD80, IL15RA, ITGA2, IL1R1	MSR1, CXCR4, CD14, HIORA, 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, CXCLU, APOL1, APOL3, FADD	CCL18, MS4A4A, CLEC7A, SLC02R1, MS4A6A, CCL13, CCL23, P2RY13, CCL16, CXCL12, CLECIOA, CCL27, CCL22, GAS7, CLEC4 M

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

TF transcription factors

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

CLASSES	Foam cells from healthy 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

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].

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, and clinical trials.

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