Transcriptome-based identification of new anti-anti-inflammatory and vasodilating properties of the n-3 fatty acid docosahexaenoic acid in vascular endothelial cell under proinflammatory conditions

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Abstract

Scope

High intakes of n-3 fatty acids exert anti-inflammatory effects and cardiovascular protection, but the underlying molecular basis is incompletely defined. By genome-wide analysis we searched for novel effects of docosahexaenoic acid (DHA) on gene expression and pathways in human vascular endothelium under pro-inflammatory conditions.

Methods and Results

Human umbilical vein endothelial cells were treated with DHA and then stimulated with interleukin(IL)-1β. Total RNA was extracted, and gene expression examined by DNA microarray. DHA alone altered the expression of 188 genes, decreasing 92 and increasing 96. IL-1β changed the expression of 2031 genes, decreasing 997 and increasing 1034. Treatment with DHA before stimulation significantly affected the expression of 116 IL-1β-deregulated genes, counter-regulating the expression of 55 genes among those decreased and of 61 among those increased. Functional and network analyses identified immunological, inflammatory and metabolic pathways as the most affected. Newly identified DHA-regulated genes are involved in stemness, cellular growth, cardiovascular system function and cancer, and included cytochrome p450 4F2(CYP4F2), transforming growth factor(TGF)-β2, Cluster of Differentiation (CD)47, caspase recruitment domain(CARD)11 and phosphodies- terase(PDE)5α.
Conclusions

Endothelial exposure to DHA regulates novel genes and related pathways. Such unbiased identification should increase our understanding of mechanisms by which n-3 fatty acids affect human diseases.

Introduction

Numerous observational and intervention studies have proven beneficial effects of the n-3 polyunsaturated fatty acids (PUFAs) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in inflammatory diseases, including atherosclerosis [1], and cancer [2]. The vascular endothelium is altered in the initiation and development of atherosclerosis [3], and in the aberrant angiogenesis [4] occurring in plaque instability, diabetes, and solid cancer [5], and appears to be an important target for such effects [6].

Although several recent studies have demonstrated that n-3 PUFAs may positively affect endothelial dysfunction [7], a comprehensive evaluation of the endothelial genomic effects exerted by n-3 PUFAs is lacking [8]. Microarray analysis, the high-throughput genomic tool that allows the simultaneous comparison of thousands of genes, is a useful tool to investigate pathophysiological mechanisms involved in a variety of human diseases, including atherosclerosis [9], and cancer [10], in a fashion not biased or restricted by a priori hypotheses. Cluster analysis of resulting data on transcriptional profiling also has the potential of revealing distinctive patterns of gene expression, thus providing novel information on the influence of n-3 PUFAs in vascular pathophysiology.

With this background, we here assessed by microarray analysis the global pattern of changes in gene expression occurring in cultured human endothelial cells exposed to DHA under pro-inflammatory conditions.

Materials and Methods

Materials

DHA (22:6 n-3 all cis) was obtained as 99% pure sodium salts from Nu-Chek (Elysian, MN, USA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

For further details, see S1 File.

Cell isolation and culture

Human umbilical vein endothelial cells (HUVECs) were harvested and maintained as described previously [11]. In particular, human cells were obtained from discarded umbilical veins, and treated anonymously, conforming with the principles outlined in the Declaration of Helsinki. The authors did not collect the tissues themselves, and cells were anonymized before use by the authors. As such, approval from the University Ethics Review Board was not necessary (see ref. [12] for previous use of such internal rule). For further details, see S1 File.

Experimental design and RNA extraction

HUVECs were preincubated with 50 μmol/L DHA for 48 h, followed by stimulation with 5 ng/mL interleukin (IL)-1β, for additional 0–3 h, after which time cells were collected and total RNA extracted using the Qiagen RNeasy kit (Qiagen, Milan, Italy) according to manufacturer’s
instructions. Concentration and purity of RNA was determined by NanoDrop ND-1000 UV-Vis Spectrophotometry (NanoDrop Technologies, Wilmington, DE, USA). No variation in the total RNA yield was observed under the different experimental conditions tested (data not shown).

**Microarray analysis**

For microarray analysis, RNAs were labeled and hybridization performed using the Gene Expression Hybridization kit (Agilent Technologies Inc., Santa Clara, CA, USA) following the manufacturer’s instructions. Gene expression profiles were generated using the 4x44K glass slide Whole Human Genome Oligo Microarray G4112A (Agilent Technologies). Each array assessed total RNAs from treated endothelial cells (DHA, IL-1 or DHA + IL-1), with RNA obtained from control endothelial cells (untreated endothelial cells). Raw data were processed by the GeneSpring 10 software (Agilent Technologies), as previously described [13]. Microarray data were made public by reporting them in the Gene Expression Omnibus (GEO) public database. The accession number is: GSE57825. For further details, see S1 File.

**Network identification and canonical pathway analysis**

Lists of genes significantly regulated by DHA and/or IL-1β were analyzed by the Ingenuity Pathways Analysis (IPA) software (Ingenuity Systems, Redwood City, CA, USA). For further details, see the S1 File.

**Real-time PCR analysis**

To validate microarray data, quantitative real-time PCR (qRT-PCR) was performed on the same samples used for microarrays experiments and on additional samples obtained under the same experimental conditions. Primer designs were based on the entire coding region for each gene (see Table A in S2 File). For further details, see S1 File.

**Protein extraction and Western blotting**

In order to verify whether the identified changes in gene expression translate in modulation of the corresponding protein HUVEC were preincubated with 50 μmol/L DHA for 48 h, followed by stimulation with 5 ng/mL IL-1β for additional 0–24 h. After this time cells were lysed and processed by sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). For further details, see S1 File.

**Knockdown of CD47 and CARD11 by small interfering RNA(siRNA)**

Gene knockdown experiments were performed by transient transfection, exposing HUVECs to a pool of pre-designed siRNA (Qiagen) against CD47 (FlexiTube siRNA: Hs_CD47_6, 7, 8), CARD11 (FlexiTube siRNA: Hs_CAR11_1, 4, 7) or to a scrambled sequences (AllStar Negative Control siRNA, 1027281, Qiagen) using the DharmaFECT Transfection Reagent (Dharmacon, CO, USA) according to the manufacturer’s protocol. For further details, see S1 File.

**Statistical analysis and comparisons**

Raw data were processed with the GeneSpring 10 software (Agilent Technologies), and differentially expressed RNA were identified using the Benjamini and Hochberg False Discovery Rate (FDR), with a P value for significance at set at 0.05. Student’s t-test for paired observations was used for comparisons of qRT-PCR results. Data are expressed as fold change (FC).
Down-regulated genes were defined as \( FC < 1.5 \), and up-regulated genes as \( FC > 1.5 \). P values of 0.05 were accepted to indicate statistically significant differences.

**Results**

**Global characterization of gene expression changes**

We had previously shown that DHA, starting from 25 \( \mu \text{mol/L} \) for 48 h, inhibited endothelial adhesion molecule expression, without endothelial toxicity, in a setting compatible with that of maximal DHA uptake and incorporation into endothelial cell membrane phospholipids [14]. All experiments were therefore performed accordingly. Comparative analysis of DHA-treated cells and untreated control cells identified that less than 1% of interrogated genes are significantly regulated, half of which (96 genes) were increased in expression and the rest (92 genes) were decreased (Fig 1). In contrast, 3 h stimulation with IL-1\( \beta \) 5 ng/mL affected a much higher number of genes, with 6.5% of the assayed gene sequences resulting in significant changes, with increased expression of 1034 genes and decreased expression of 997 other genes (Fig 1).
Globally, treatment with DHA before IL-1β stimulation altered the expression of more than 2% of the IL-1β regulated genes (Fig 1).

**Functional categorization of DHA- and IL-1β-regulated genes**

**Effects of DHA on endothelial baseline gene expression.** The categorization of regulated genes according to their function showed that DHA per se, compared with control conditions (absence of any treatment), regulated the expression of genes mainly associated with biological processes including cancer, tissue morphology, antigen presentation, cell-to-cell signaling and interaction, and cell-mediated immune response (Table B in S2 File). Although with lower scores, categories of cardiovascular system development and function, as well as cardiovascular disease also resulted significantly affected by DHA (Table C in S2 File). Among these biological processes, the largest sets of genes modulated by DHA were functionally related to hematological neoplasms and mammary tumors (13 and 14 molecules, respectively), blood vessel remodeling, and cardiovascular tissue morphology (5 and 4 molecules, respectively), cell activation (14 molecules), leukocyte movement (10 molecules), regulation of blood pressure and cardiovascular system morphology (7 and 10 molecules, respectively). The most cited gene of cardiovascular interest were transforming growth factor (TGF)-β2, angiopoietin (ANGPT)-1 and CD47, the expression of which was reduced by 34%, 40%, and 35% respectively (with FC of 1.51, 1.67, 1.54, P<0.05% vs control) and cytochrome P 450 (CYP)4F2, increased by 152%, with a FC of 2.52 (P<0.05% vs control).

IPA identified several interesting pathways associated with DHA treatment (Fig 2). The top canonical pathways, based on their significance (P value), included: role of Nanog in mammalian embryonic stem cell pluripotency, leukocyte extravasation signaling, tight junction signaling, and regulation of interleukin(IL)-2 expression in activated and anergic T lymphocytes. Genes included in each group of the top ten signaling pathway are listed in Table D in S2 File. When IPA was inquired for network analysis, it yielded 11 significant regulatory networks with a score >2. The number one network here ranked (score = 44, focus molecules = 22) was associated with cell movement, hematological system development and function, and immune cell trafficking (S1 Fig). Top functions of the subsequent 4 highly significant networks included cancer, lipid metabolism and cardiovascular disease.

**Effect of DHA on the expression of IL-1β-regulated genes.** Cell stimulation with IL-1β changed the expression of genes mainly associated with cellular development, cell death and survival, the development and function of cardiovascular and hematological systems, and tissue morphology (Table E in S2 File). Among these, most genes modulated by IL-1β appeared to be related to cell differentiation (283 genes), tumor cell proliferation (178 genes), apoptosis (315 genes), and the development of cardiovascular system and blood vessels (168 and 135 genes, respectively). Results of canonical pathway analysis again revealed several pathways as significantly associated with IL-1β stimulation (S2 Fig). The top 10 canonical pathways, ranked by their significance (P value) included: the role of IL-17A in arthritis, the activation of hepatic fibrosis/hepatic stellate cell, the role of protein kinase receptor (PKR) in interferon induction and antiviral response, the role of macrophages, fibroblasts and endothelial cells in rheumatoid arthritis, and the role of IL-17F in allergic inflammatory airway diseases.

To evaluate whether and how DHA affects the endothelial inflammatory response as by IL-1β, we compared the gene expression profile of HUVECs treated with DHA and then stimulated with IL-1β with those of HUVECs only stimulated with IL-1β. Here we selected, among genes regulated by DHA + IL-1β vs IL-1β, only those for which expression values diverged by at least of 1.5 FC from IL-1β (Tables 1 and 2).
When classified by their function, these genes resulted associated with biological processes including cancer, cell growth and proliferation, nervous system development and function, tissue development, and cardiovascular system development and function. Among such biological processes, most genes affected by DHA were related to breast and digestive system cancer (32 molecules), cell proliferation (28 molecules), and morphology of the cardiovascular system (10 molecules) (Table F in S2 File). IPA identified assorted canonical pathways associated with these genes. Ranked according to their significance (P value), they included: pigment epithelium-derived factor (PEDF) signaling, regulation of IL-2 expression in activated and anergic T lymphocytes, ultra-violet A (UVA)-induced MAPK signaling, interferon signaling, the role of tissue factor in cancer, proliferation-inducing ligand (APRIL)-mediated signaling, and p38 MAPK signaling (Fig 3). Genes included in each group of the top ten signaling pathway are
| Probe Name | FC (IL-1β vs None) | Regulation | FC (DHA plus IL-1β vs IL-1) | Regulation | Symbol     | Description                                      | Genbank Accession |
|------------|------------------|------------|-----------------------------|------------|------------|--------------------------------------------------|------------------|
| A_24_P687  | 1.73             | down       | 1.5742                      | up         | CDH6       | Cadherin 6                                       | NM_004932        |
| A_24_P221154 | 1.30           | down       | 1.6453                      | up         | ABCD3      | ATP-binding cassette                              | NM_002858        |
| A_24_P100130 | 1.10            | down       | 1.7559                      | up         | BCL2L1     | BCL2-like 1                                      | NM_001191        |
| A_23_P120354 | 1.38            | down       | 1.5307                      | up         | ANKRDS7    | Ankyrin repeat domain 57                         | NM_023016        |
| A_24_P98109 | 1.84             | down       | 1.5377                      | up         | SNX10      | Sorting nexin 10                                 | NM_013322        |
| A_24_P7600  | 1.09             | down       | 1.8849                      | up         | FBXL7      | F-box and leucine-rich repeat protein 7           | NM_012304        |
| A_23_P67278 | 1.54             | down       | 1.8525                      | up         | ZNF443     | Zinc finger protein 443                          | NM_005815        |
| A_23_P149345 | 2.71            | down       | 1.56583                     | up         | PTPN22     | Protein tyrosine phosphatase                      | NM_015967        |
| A_23_P50399 | 1.22             | down       | 1.5416                      | up         | DCAF15     | DDB1 and CUL4 associated factor 15                | NM_138353        |
| A_24_P256830 | 1.18            | down       | 1.6193                      | up         | EXOC5      | Exocyst complex component 5                      | NM_006544        |
| A_23_P360769 | 1.06            | down       | 1.5956                      | up         | MAN2A1     | Mannosidase                                      | NM_002372        |
| A_24_P258473 | 1.13            | down       | 1.9839                      | up         | SMOC1      | SPARC related modular calcium binding 1           | NM_001034852     |
| A_23_P40240 | 1.32             | down       | 2.7139                      | up         | CTSZ       | Cathepsin Z                                      | NM_001336        |
| A_23_P214011 | 2.14            | down       | 1.7433                      | up         | CDH6       | Cadherin 6                                       | NM_004932        |
| A_24_P62659 | 2.08             | down       | 1.6252                      | up         | TSPAN2     | Tetraspanin 2                                    | NM_005725        |
| A_24_P147910 | 1.03            | down       | 1.6621                      | up         | SEPT9      | Septin 9                                         | NM_006640        |
| A_24_P363408 | 5.16            | down       | 1.7213                      | up         | HEY2       | Hair/elancer-of-split related with YRPW motif 2   | NM_012259        |
| A_23_P32577 | 2.35             | down       | 1.5450                      | up         | DACH1      | Dachshund homolog 1 (Drosophila)                  | NM_080759        |
| A_23_P356139 | 1.73             | down       | 1.5485                      | up         | FAM178A    | Family with sequence similarity 178              | NM_018121        |
| A_24_P243749 | 2.06            | down       | 1.5197                      | up         | PDK4       | Pyruvate dehydrogenase kinase                     | NM_002612        |
| A_24_P176409 | 2.16            | down       | 1.7183                      | up         | ZFP90      | Zinc finger protein 90 homolog (mouse)           | NM_133458        |
| A_24_P104538 | 2.01            | down       | 1.5197                      | up         | BMX1       | Nucleosome-remodeling factor subunit BPTF          | NM_001726698     |
| A_32_P56392 | 1.48             | down       | 1.6076                      | up         | RBM4       | RNA binding motif protein                         | NM_002139        |
| A_24_P187218 | 1.68             | down       | 1.6168                      | up         | PCDH9      | Protocadherin 9                                  | NM_020403        |
| A_23_P156907 | 2.79             | down       | 1.5262                      | up         | SOBP       | Sine oculis binding protein homolog (Drosophila)  | NM_018013        |
| A_32_P233769 | 1.07             | down       | 1.5719                      | up         | U.F.       |                                                   |                  |
| A_24_P590806 | 1.14             | down       | 1.5892                      | up         | U.F.       |                                                   |                  |
| A_32_P62835 | 1.34             | down       | 1.8267                      | up         | U.F.       |                                                   |                  |
| A_23_P146981 | 1.14             | down       | 1.7179                      | up         | U.F.       |                                                   |                  |
| A_32_P148627 | 3.82             | down       | 1.6307                      | up         | U.F.       |                                                   |                  |
| A_23_P335190 | 1.62             | down       | 1.5310                      | up         | U.F.       |                                                   |                  |
| A_32_P137408 | 1.08             | down       | 1.8703                      | up         | U.F.       |                                                   |                  |
| A_32_P45229 | 3.25             | down       | 1.8844                      | up         | U.F.       |                                                   |                  |
| A_32_P55799 | 1.03             | down       | 1.8320                      | up         | U.F.       |                                                   |                  |
| A_32_P225328 | 1.78             | down       | 1.6220                      | up         | U.F.       |                                                   |                  |
| A_32_P214969 | 1.17             | down       | 2.1562                      | up         | U.F.       |                                                   |                  |
| A_32_P190222 | 2.05             | down       | 1.9090                      | up         | U.F.       |                                                   |                  |
| A_24_P200427 | 1.28             | up         | 1.5251                      | down       | PAICS      | Phosphoribosylamineimidazole carboxylase          | NM_01079525      |
| A_32_P51524 | 1.63             | up         | 2.4827                      | down       | LOC595101  | mRNA; cDNA DKFP686H21113                         | CR627362         |
| A_23_P77721 | 1.00             | up         | 1.6413                      | down       | LOC100131601| cDNA FLJ45059 fs                                | AK127004         |
| A_24_P927444 | 1.66             | up         | 1.6194                      | down       | CYTSB      | Sperm antigen with calponin homology and coiled-coil domains 1 | BC033618        |

(Continued)
When IPA was enquired for network analysis, it yielded 6 significant regulatory networks (score > 2). The first ranked network (score = 38, focus molecules = 18) (S3 Fig) was associated with cellular function and maintenance, humoral immune response and protein synthesis. Top functions of the other 5 highly significant networks were associated with drug metabolism, cell death and survival, organ morphology and cardiovascular system development and function.

Table 1. (Continued)

| Probe Name | FC (IL-1 vs None) | Regulation | FC (DHA plus IL-1 vs IL-1) | Regulation | Symbol | Description | Genbank Accession |
|------------|------------------|------------|-----------------------------|------------|--------|-------------|------------------|
| A_32_P72110 | 2.10 up | down | 1.8706 | PVR | Poliovirus receptor | NM_006505 |
| A_24_P319369 | 1.74 up | down | 1.6563 | F11R | F11 receptor | NM_016946 |
| A_23_P363344 | 1.14 up | down | 1.5518 | TPM1 | Tropomyosin 1 (alpha) | NM_000366 |
| A_23_P500844 | 5.60 up | down | 1.9049 | PDE5α | Phosphodiesterase 5α | NM_001083 |
| A_24_P145035 | 1.25 up | down | 1.6981 | | | |
| A_23_P338479 | 2.73 up | down | 1.5364 | CD274 | CD274 molecule | NM_014143 |
| A_32_P115701 | 1.22 up | down | 1.6080 | NARG2 | NMDA receptor regulated 2 | NM_024611 |
| A_32_P7974 | 1.73 up | down | 1.6001 | TDRD10 | Tudor domain containing 10 | NM_182499 |
| A_23_P22566 | 1.18 up | down | 1.5171 | IFIT1 | Interferon-induced protein with tetratricopeptide repeats 1 | NM_001548 |
| A_24_P416997 | 3.12 up | down | 1.6076 | APOL3 | Apolipoprotein L | NM_145641 |
| A_24_P945262 | 1.55 up | down | 2.7229 | CARD11 | | AK097139 |
| A_32_P215143 | 4.40 up | down | 4.7029 | LOC100288583 | Hypothetical protein LOC100288583 | XM_002343463 |
| A_24_P148261 | 2.07 up | down | 2.0703 | TGF-β2 | Transforming growth factor-β2 | NM_001135599 |
| A_23_P35995 | 1.93 up | down | 1.9822 | ASAM | Adipocyte-specific adhesion molecule (ASAM) | NM_024769 |
| A_32_P52823 | 1.52 up | down | 1.5729 | VPS41 | | BX648347 |
| A_23_P343927 | 2.43 up | down | 1.5286 | HIST2H2AB | Histone cluster 2 | NM_175065 |
| A_32_P8351 | 1.74 up | down | 2.2334 | LOC595101 | mRNA; cDNA DKFZp686H21113 | CR627362 |
| A_24_P829261 | 1.00 up | down | 3.1194 | MALAT1 | Metastasis associated lung adenocarcinoma transcript 1 | NR_002819 |
| A_32_P62769 | 1.59 up | down | 1.7918 | LOC100288933 | PREDICTED: hypothetical LOC100288933 | XR_078366 |
| A_23_P211080 | 2.99 up | down | 1.6303 | IFNAR2 | Interferon alpha receptor | NM_207585 |
| A_23_P59691 | 1.53 up | down | 1.6471 | PAX4 | Paired-box transcription factor | AF43978 |
| A_24_P11737 | 1.06 up | down | 1.6010 | LOC440894 | cDNA FLJ31522 | AK056084 |
| A_24_P406132 | 1.04 up | down | 1.6177 | MAPK13 | mitogen-activated protein kinase 13 | NM_002754 |
| A_24_P942703 | 3.54 up | down | 2.8578 | LOC728153 | cDNA FLJ11903 | AK021965 |
| A_24_P450372 | 1.37 up | down | 2.0113 | U.F. | | |
| A_24_P937582 | 1.20 up | down | 1.9464 | U.F. | | |
| A_24_P450493 | 5.08 up | down | 1.5469 | U.F. | | |
| A_23_P22978 | 1.29 up | down | 1.5873 | U.F. | | |
| A_24_P938516 | 1.08 up | down | 1.5411 | U.F. | | |
| A_24_P649829 | 1.22 up | down | 1.5841 | U.F. | | |
| A_24_P59485 | 2.58 up | down | 1.6852 | U.F. | | |

F.C. = fold change; U.F. = Genes of unknown function.

Genes regulated antagonistically with IL-1β.

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## Table 2. Genes differentially expressed in DHA plus IL-1β- versus IL-1β-treated cells.

| Probe Name   | FC (IL-1 vs None) | Regulation | FC (DHA plus IL-1 vs IL-1) | Regulation | Symbol  | Description                                           | Genbank Accession |
|--------------|------------------|------------|----------------------------|------------|---------|-------------------------------------------------------|-------------------|
| A_23_P139339 | 1.12             | down       | 1.7013                     | down       | PAAF1   | Proteasomal ATPase-associated factor 1                | NM_025155         |
| A_23_P58117  | 1.76             | down       | 1.9171                     | down       | SHROOM3 | Shroom family member 3                                | NM_020859         |
| A_24_P237601 | 2.28             | down       | 1.5399                     | down       | RPS6KA5 | Ribosomal protein S6 kinase                          | NM_182398         |
| A_23_P87013  | 1.12             | down       | 1.6039                     | down       | TAGLN   | Transgelin                                            | NM_001001522      |
| A_32_P129752 | 1.57             | down       | 1.7424                     | down       | TMEM30B | Transmembrane protein 30B                            | NM_001017970      |
| A_23_P57547  | 1.16             | down       | 1.5709                     | down       | SLC25A17| Solute carrier family 25                             | NM_006358         |
| A_23_P70007  | 1.08             | down       | 1.6774                     | down       | HMMR    | Hyaluronan-mediated motility receptor (RHAMM)          | NM_012484         |
| A_23_P6818   | 1.14             | down       | 1.6614                     | down       | SEMA3G  | Sema domain                                           | NM_020163         |
| A_23_P113393 | 1.68             | down       | 1.5961                     | down       | APLN    | Apelin                                                | NM_017413         |
| A_23_P135990 | 1.44             | down       | 1.6862                     | down       | SLC02A1 | Solute carrier organic anion transporter family       | NM_005630         |
| A_23_P127891 | 1.66             | down       | 1.6109                     | down       | BDNF    | Brain-derived neurotrophic factor                     | NM_170735         |
| A_32_P181061 | 2.39             | down       | 2.1453                     | down       | U.F.     |                                                       |                   |
| A_23_P434040 | 2.23             | down       | 2.4356                     | down       | U.F.     |                                                       |                   |
| A_32_P53183  | 1.59             | down       | 1.5028                     | down       | U.F.     |                                                       |                   |
| A_24_P297302 | 2.53             | down       | 1.7212                     | down       | U.F.     |                                                       |                   |
| A_24_P84370  | 1.02             | down       | 1.5995                     | down       | U.F.     |                                                       |                   |
| A_23_P215505 | 1.90             | up         | 2.6764                     | up         | RAPGEF5 | Rap guanine nucleotide exchange factor (GEF) 5        | NM_012294         |
| A_24_P133933 | 1.11             | up         | 1.7661                     | up         | ARHGDIA | Rho GDP dissociation inhibitor (GDI) alpha            | NM_004309         |
| A_24_P525917 | 1.57             | up         | 1.5075                     | up         | NFATC2  | Nuclear factor of activated T-cells                   | Q13469            |
| A_24_P234838 | 1.37             | up         | 1.5085                     | up         | PCDH1   | Protocadherin 1                                      | NM_032420         |
| A_24_P366994 | 1.03             | up         | 1.9132                     | up         | ARHGDIA | Rho GDP dissociation inhibitor (GDI) alpha            | NM_004309         |
| A_23_P169738 | 1.19             | up         | 1.5391                     | up         | SOX7    | SRY (sex determining region Y)-box 7                  | NM_031439         |
| A_23_P218807 | 2.23             | up         | 1.7046                     | up         | ZC3H7B  | Zinc finger CCCH-type containing 7B                   | NM_017590         |
| A_23_P96165  | 1.04             | up         | 1.6745                     | up         | C11orf80| Chromosome 11 open reading frame 80                  | NM_024650         |
| A_23_P202435 | 1.02             | up         | 1.6325                     | up         | ADD3    | Adducin 3 (gamma)                                    | NM_016824         |
| A_32_P165477 | 1.50             | up         | 1.5528                     | up         | SLC7A11 | Solute carrier family 7                               | NM_014331         |
| A_32_P55241  | 2.88             | up         | 1.5342                     | up         | SHISA2  | Shisa homolog 2 (Xenopus laevis)                      | NM_001007538      |
| A_23_P112078 | 1.75             | up         | 1.5282                     | up         | MFHAS1  | Malignant fibrous histiocytoma amplified sequence 1   | NM_004225         |
| A_32_P20004  | 1.58             | up         | 1.7230                     | up         | U.F.     |                                                       |                   |
| A_24_P911327 | 1.03             | up         | 1.5692                     | up         | U.F.     |                                                       |                   |
| A_32_P62480  | 1.25             | up         | 1.9490                     | up         | U.F.     |                                                       |                   |
| A_32_P222250 | 1.53             | up         | 1.6226                     | up         | U.F.     |                                                       |                   |
| A_32_P201976 | 1.43             | up         | 1.7541                     | up         | U.F.     |                                                       |                   |
| A_32_P48149  | 1.13             | up         | 1.5691                     | up         | U.F.     |                                                       |                   |
| A_24_P366989 | 1.01             | up         | 1.8311                     | up         | U.F.     |                                                       |                   |
| A_32_P229365 | 1.19             | up         | 1.6773                     | up         | U.F.     |                                                       |                   |
| A_24_P85619  | 1.16             | up         | 1.5773                     | up         | U.F.     |                                                       |                   |
| A_32_P30898  | 1.08             | up         | 1.5220                     | up         | U.F.     |                                                       |                   |
| A_32_P174374 | 1.02             | up         | 2.0840                     | up         | U.F.     |                                                       |                   |

(Continued)
CD47 and CARD11 functional analysis

To best define the functional role of some genes affected by DHA in endothelial cells, we elected to silence the expression of two of them, CARD11 and CD47, and then to evaluate the
resulting effects in terms of expression of genes potentially correlated. We selected CARD11 and CD47 because both were hypothesized to be involved in the signaling pathway leading to the activation of the pro-inflammatory master transcriptional factor nuclear factor (NF)-κB [15]. Transfections with siRNAs neither affected cell morphology, nor produced overt signs of endothelial cell damage (S4A and S4B Fig). Analysis of CD47 and CARD11 gene expression after silencing showed a significant and specific decrease in the levels of both mRNAs, as analyzed by qRT-PCR, by >70% and >80% under basal and stimulated condition for CD47 and CARD11, respectively (S4C Fig). Under the same conditions the levels of several pro-inflammatory and vascular remodeling genes were assessed. CARD11 silencing blunted the IL-1β-mediated expression of most pro-inflammatory genes tested, including vascular cell adhesion molecule (VCAM)-1, intercellular adhesion molecule (ICAM)-1, and E-selectin (E-Sel) (S5 Fig), likely through an attenuation of the NF-κB signaling pathways [16]. Interestingly, CARD11 knockdown also downregulated the expression of genes encoding for vascular endothelium growth factor (VEGF) and matrix metalloproteinase (MMP)-2, and increased the expression of the potent anti-inflammatory cytokine IL-10 [17] (S5 Fig), thus disclosing new unexpected functions for this gene. Finally, we observed that CD47 knockdown reduced the expression of all the pro-inflammatory, pro-angiogenic genes tested, confirming in our experimental conditions the pro-inflammatory role previously ascribed to this molecule [18] (S6 Fig).

Microarray analysis verification and validation

We used qRT-PCR and Western analysis to validate the microarray results for a subset of genes selected from the DHA- plus IL-1β and the DHA+ plus IL-1β experimental conditions. Target genes were chosen based upon their potential vascular regulatory functions. Among those deregulated by DHA under resting conditions, we focused our attention on the modulation of CD47 and CYP4F2 expressions. For these two genes, microarray analysis showed a down-regulation by 1.5 FC and an up-regulation by 2.52 FC, respectively. In agreement with microarray data, qRT-PCR confirmed the reduction of CD47 and the increase of CYP4F2 mRNA expressions (-40 ± 9% and +30 ± 5%, respectively, for 50 μmol/L DHA; p<0.01). Western analysis confirmed such findings in terms of protein expression (Fig 4A). Exposure of HUVECs to IL-1β induced the expression of several well-known pro-inflammatory genes, among those encoding for VCAM-1 (27.7 FC), for the chemokine (C-C motif) ligand (CCL)2 (also referred to as monocyte chemotactic protein (MCP)-1) (3.78 FC) and the prostaglandin-endoperoxide synthase (PTGS)-2 (also known as cyclooxygenase (COX)-2) (1.8 FC), in addition to a vast series of new unexplored genes of cardiovascular interest, such as those encoding for transforming growth factor (TGF)-β2 (2.2 FC) and phosphodiesterase (PDE)5α (5.6 FC). Analysis by qRT-PCR confirmed microarray results showing the induction of VCAM-1, MCP-1, COX-2, TGF-β2 and PDE5α upon IL-1β stimulation by 400-, 6.5-, 6-, 5- and 30-fold, respectively. We also confirmed microarray results obtained in cells exposed to DHA before IL-1β stimulation in comparison with IL-1β stimulation alone. Under these conditions, microarray analysis showed, for the DHA plus IL-1β condition, a down-regulation of TGF-β2, PDE5α and CARD11 by 2.0 and 1.9 and 2.72 FC, respectively. Such expression changes were confirmed at both qRT-PCR (-35%, -37% and -25%; respectively, S7 Fig) and Western analysis (Fig 4B).

We noticed that several other NF-κB-target genes, including VCAM-1, MCP-1 and COX-2, robustly induced by IL-1β and previously described as affected by DHA [14, 19], did not result down-regulated by DHA in the present microarray analysis. It must however be recognized that conclusions drawn from the comparison of gene expressions under two treatments with
this technique are strictly dependent upon the selection of significance criteria. In microarray analyses, these are usually set at values aimed at achieving a robust degree of specificity at the price of sensitivity. Therefore it is well possible that genes the expression of which does not achieve the pre-defined threshold are here classified as unchanged, when in reality expression level changed. In our analysis this was true, for example, for VCAM-1, MCP-1, COX-2, ICAM-1 and E-Sel, the induced expression of which, even resulting as not affected by DHA at the microarray analysis, still resulted down-regulated at qRT-PCR analysis (S7 Fig), a technique

Fig 4. Validation of gene expression changes in DHA-treated HUVECs by Western blotting. (A) HUVECs were treated with DHA for 48 h, after which time total protein were extracted and subjected to Western analysis using an anti-CD47 and an anti-CYP4F2 antibody. As control for equal loading, blots were stripped and re-probed with an anti-β-actin antibody. CD47 and CYP4F2 representative immunoblots are shown in the lower panels, while densitometric analysis for the CD47 and CYP4F2, normalized to β-actin and expressed as fold induction over basal control, are shown in the upper panel. Bars represent mean±SD (n = 3). *P<0.05 vs basal control. (B) HUVECs were treated with/without DHA for 48 h, and then stimulated with IL-1β. After 24 h, total protein were extracted and subjected to Western analysis using antibodies against PDE5α, TGF-β2 and CARD11. As control for equal loading, blots were stripped and re-probed with an anti-β-actin antibody. Representative immunoblots are shown in the lower panels, while densitometric analysis for each protein, normalized to β-actin and expressed as fold over basal control, are shown in the upper panel. Bars represent mean±SD (n = 3). *P<0.05 vs IL-1β.
widely recognized as of greater sensitivity than microarray [20, 21], and here run in parallel with a few a priori selected genes.

Discussion
This study reports on the changes in global gene expression in human endothelial cells exposed to DHA under resting and pro-inflammatory conditions. Besides confirming gene regulations previously assessed with a classical candidate-gene approach, this analysis discloses previously unsuspected genes and gene pathways affected by the exposure induced of endothelial cells to DHA, thus expanding our knowledge on biological effects of n-3 PUFAs.

Although there is still no unanimous consensus on the vasculoprotective role of n-3 PUFAs [22], a significant number of experimental studies and clinical intervention trials have shown a protective effect for these substances derived from the intake of dietary fish or fish oil [1]. However, the probably multiple molecular mechanisms by which EPA and DHA affect cardiovascular health still remain incompletely understood [1]. To obtain a comprehensive overview of the scope of biological processes modulated by n-3 PUFAs whole-genome transcriptomic analyses have been previously performed on peripheral blood mononuclear cells (PBMCs) of human subjects taking fish oils supplements, with results suggesting a modulation by n-3 PUFAs of inflammatory-, oxidative-, endoplasmic reticulum (ER) stress- and apoptosis-regulatory pathways [21, 23–27]. However, studies examining the effects of n-PUFAs on whole-genome expression of endothelial cells, which are prominent targets for n-3 PUFA effects [1], have been lacking. Our analysis on these cells had two main objectives: firstly, to explore the effects of DHA supplementation on changes in endothelial gene expression under resting conditions; and secondly, to evaluate the effects of DHA on changes in endothelial gene expression under pro-inflammatory conditions. This latter goal was accomplished comparing the gene expression profile of IL-1β-stimulated cells with those of cells treated with DHA before IL-1β stimulation.

Fixing rather stringent analytical conditions of FC and significance (cut-offs >1.5, P values <0.05), under unstimulated conditions we identified 188 genes as differentially expressed by DHA. Taking advantage of ranked gene expression pathways, the results revealed that pathways related to stemness, cell-to-cell signaling and inflammation were among those mostly differentially expressed. In particular, the canonical pathway analysis revealed the “role of Nanog in mammalian embryonic stem cell pluripotency” as the most significant signaling pathway modulated by DHA. This is an example of an unexpected and interesting result. The possibility that DHA regulates the expression of stemness transducers may provide an opportunity to use DHA and n-3 PUFAs in general to enhance the generation of progenitor cells or stem-like cells and enhance reparative functions of endothelial progenitor cells (EPCs). Despite the paucity of experimental data on the effect of n-3 PUFAs on EPC function, some recent reports support this hypothesis [28, 29].

A classification of DHA-regulated genes into functional related groups revealed that DHA per se maximally impacts gene expression connected with cancer initiation and progression (best scored function annotation). However, although less scored, we also focused our attention on activities connected with cardiovascular system development and functions. The most cited genes gathered under this category are involved in the morphology of cardiovascular system and the regulation of blood pressure, and include TGF-β2, ANGPT-1, CD47 and CYP4F2, the first three of which are down-regulated, while the last is up-regulated by DHA. The TGF-β family includes a large number of molecules structurally and functionally related, acting as multifunctional regulators of a wide range of biological processes, including morphogenesis, embryonic development, adult stem cell differentiation, immune regulation, wound healing,
inflammation, atherogenesis and cancer [30]. Despite their high sequence homology, striking differences exist between the 5' flanking regions of each such genes. TGF-β1 has not a TATAA box nor nuclear factor (NF)-κB binding sites, otherwise present in the TGF-β2 promoter sequence [31, 32]. Furthermore, while TGF-β1 and -β3 bind directly to the TGF-β receptor type II (TβRII), the binding of TGF-β2 requires the presence of a co-receptor, which may explain differences in activities of TGF-β2 and -β1 [33]. TGF-β1 is the isoform most extensively studied in relation to atherogenesis, with somewhat contrasting results [34, 35]. More pertinently to our results, it was demonstrated that TGF-β2 may specifically mediate neointimal thickening in LDLr-/- mice after carotid artery ligation [36], and induces the activation of pro-inflammatory transcription factors, such as NF-κB, in various cellular models [37]. Furthermore, a synergistic cross-talk between TGF-β2 and IL-1β in the activation of NF-κB has been also documented [38]. In line with this pro-inflammatory cross-talk, we observed that IL-1β could induce TGF-β2, but not TGF-β1 expression; and that DHA treatment reduced both the basal and the up-regulated expression of TGF-β2, thus revealing another mechanistic explanation for the anti-inflammatory activities attributed to DHA. The mechanism by which DHA may reduce basal and IL-1β mediated expression of TGF-β2 goes beyond the aim of the present report and was not investigated further. However, since DHA is known to negatively interfere with the activation of the transcription factor NF-κB in the vascular endothelium [19], the selective presence of a NF-κB binding site in the TGF-β2 promoter sequence [32] may help explaining both the preferential induction of TGF-β2 by IL-1β and the down-regulation of TGF-β2 by DHA. Previous reports investigating the effect of DHA on TGF-β2 expression are scarce. However, in line with our results, the expression of TGF-β2 was reduced in platelets of rats fed on a diet high in fish oils [39].

Interestingly, TGF-βs are extracellularly activated by the catalytic activity of thrombospondin (TSP)-1, the receptor for which, CD47, also resulted down-regulated by DHA. TSP-1/CD47 interaction has been reported to exert pro-inflammatory activities in the vascular endothelium, inducing the expression of pro-atherogenic adhesion molecules and the subsequent binding of monocytes [18] and T-cell [40], and to negatively interfere with the nitric-oxide (NO)-driven vascular smooth muscle cell (VSMC) relaxation [41], and, as a consequence, affecting local and systemic blood flow [42]. We here confirm the involvement of CD47 in the orchestration of endothelial pro-inflammatory response to IL-1β, thus expanding the findings obtained by Narizhneva at al. upon endothelial exposure to TNF-α [18]. We observed that DHA reduces the basal expression of CD47, thus potentially contributing to the small but significant decrease in blood pressure generally ascribed to fish oil intake [43]. While a down-regulating effect of DHA on TSP-1 has been recently reported in adipocyte-macrophages co-cultures [44], our investigation is the first reporting a down-regulating activity by DHA on the expression of the TSP-1 receptor CD47. Finally, among genes regulated by DHA under basal conditions, we observed the up-regulation of CYP4F2, a member of the cytochrome P 450 gene 4 family. In general, human CYP comprises a superfamily of heme-thiolate proteins that play critical roles in the metabolism of endogenous as well as xenobiotic-derived molecules [45]. Families from 1, 2 and 3 are known to be involved in the epoxidation of drugs and of other xenobiotics in the liver [46], whereas the CYP4 –CYP4Fs in particular— are known to n-hydroxylate a variety of long-chain unsaturated and branched-chain FA, vitamins with long alkyl side chains, leukotrienes (LT), prostaglandins (PG) and hydroxyeicosatetraenoic acids (HETE) [46]. CYP4F was first discovered to metabolize and inactivate the pro-inflammatory leukotriene (LT) B4 [47]. Recent reports have shown the expression of CYP4F2 and CYP4F3 genes in the colon of patients with inflammatory celiac disease [48]. In particular, the CYP4F3 gene resulted overexpressed during cryptic hyperplasia, while in remission stages a strong increase in CYP4F2 gene expression was observed [49]. These data suggest that CYP4F3 is involved in promoting colon
inflammation, while the increased expression of CYP4F2 would mediate disease resolution. CYP4F2 has been also recognized to metabolize arachidonic acid (AA) to 20-HETE, a molecule endowed with anti- and pro-hypertensive properties [50]. Since DHA may be used by CYP4F2 as substrate alternative to AA [51], DHA may exert anti-inflammatory and anti-hypertensive effects by both decreasing the AA conversion to 20-HETE and decreasing LTB4 levels. The ability of DHA to interfere with the CYP4F-metabolic pathway through genomic ways is exciting, and deserves further investigations.

IL-1β has long been considered to play a central role in orchestrating the various cellular changes that contribute to inflammation in atherogenesis [52]. In humans IL-1β expression is higher in atherosclerotic lesions than in normal arteries [53]. Studies in mouse models of atherosclerosis have shown a reduced atherosclerotic burden in IL-1β-deficient animals [54]. For these reasons we chose to challenge the endothelium with IL-1β as an inflammatory trigger to evaluate the interference by DHA with endothelial activation. In agreement with previous DNA microarray studies [55–57], the gene expression profile analysis here revealed complex inflammatory responses comprised of up- and down-regulation of genes connected with several cellular processes, including cell development, cell death and survival, and cardiovascular system development and function (this last as the 5th best scored change). Among the up-regulated genes, our microarray data confirm well-validated changes including COX-2, IL-8, MCP-1, IL-6, E-selectin, VCAM-1, ICAM-1, and fractalkine, all reported as IL-1β up-regulated genes in previous microarray studies [56–59].

As a second aim of our study, among the confirmed IL-1β-up-regulated genes, we then focused our attention on those of relevance for atherosclerosis and maximally counter-impacted by DHA treatment. Some of these genes were grouped in known canonical pathways, such as TGF-β2 and PDE5 associated with protein kinase A signaling; and CARD11, associated with the regulation of IL-2 expression in activated and anergic T Lymphocytes; while others, such as F11R, were not connected with any canonical pathways.

F11R, also known as junctional adhesion molecule (JAM)-A, is a small multifunctional immunoglobulin expressed by platelets, leukocytes, endothelial and epithelial cells, acting as a ligand for the integrin lymphocyte function-associated antigen (LFA)-1 during leukocyte transmigration [60], a platelet receptor [61], and a receptor for rheoviruses [62]. A prominent role for F11R in several inflammatory pathologic processes, including skin inflammation, meningitis, peritonitis, liver and myocardial ischemia, and coronary artery disease, has been recently recognized [63]. In particular, a significant increase in the level of soluble F11R has been found in the serum of patients with coronary artery disease [64]; and significantly higher levels of the F11R mRNA and protein are expressed within unstable atherosclerotic plaques in association with the endothelium and platelets [65], suggesting the involvement of F11R in atherothrombosis. In vitro and in vivo studies have shown that, under physiological conditions, a non-activated, healthy endothelium expresses low levels of F11R mRNA, and in this case the F11R/JAM-A protein resides primarily within the endothelial tight junctions [66]. When endothelial cells are exposed to pro-inflammatory cytokines, such as IL-1β and tumor necrosis factor (TNF)α, mRNA and protein levels for F11R rise significantly, followed by the insertion of newly-synthesized F11R molecules into the endothelial plasma membrane facing the vessel lumen [67], thus providing the endothelium with new pro-thrombotic and pro-adhesive properties. In agreement with previous data [68], we observed an increase in F11R mRNA expression upon IL-β stimulation. We also observed that DHA pretreatment antagonizes IL-1β-mediated increase in mRNA levels of F11R, an anti-inflammatory effect never reported before, highlighting potentially new mechanistic explanations for the anti-atherogenic and anti-thrombotic effects of DHA. The presence of NF-κB binding sites in the promoter of the F11R gene [69] may explain the down-regulating effect exerted by DHA, confirming for DHA the
repeatedly described interference with the expression of NF-κB target genes [19]. NF-κB activation indeed represents one of the foremost mechanisms responsible for IL-1β-induced endothelial activation. Similarly to many activators of NF-κB, this occurs through the so-called canonical pathway, whereby activation depends on stimulation of the IκB kinase (IKK) complex. IKK in turn orchestrates the phosphorylation and subsequent degradation of IκB, a protein that sequesters NF-κB (particularly in the form of RelA/p65) in the cytoplasm. Upon IκB degradation, NF-κB is then free to translocate to the nucleus and stimulate transcription of various pro-inflammatory genes [15, 70]. In our previous work on the mechanisms by which DHA interferes in the signaling leading to NF-κB activation, we have shown that DHA inhibits at least two molecular switches involved in NF-κB activation, namely NAD(P)H oxidase and PKCε [19]. The present data suggest an additional level of interference in the signaling pathway leading to NF-κB activation. We here observed that IL-β induced, and DHA counter-reduced, the expression of the Caspase recruitment domain (CARD)11 mRNA, which encodes for a scaffold protein involved, upon trimerization with B-cell lymphoma protein 10 (Bcl10) and Mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1), in the activation of IKK [71]. In spite of generally reported tissue-specific expression of CARD11 in hematopoietic cells [16], a recent gene expression analysis has recognized the expression of CARD11 also in endothelial cells and its induction in the endothelium within a hemangioma [72]. To the best of our knowledge, this is the first report describing an induction of CARD11 by IL-1β in endothelial cells and its direct involvement in the induction of pro-inflammatory genes. The down-regulation of IL-1β-induced expression of CARD11 by DHA is therefore an additional mechanistic explanation underpinning the anti-inflammatory effect exerted by n-3 PUFAs. Endothelial dysfunction, reflected by reduced NO availability, is recognized as a causal factor in promoting atherosclerosis [73]. Synthesized from L-arginine through the catalysis of a family of NO synthase isoforms, NO activates the soluble guanylate cyclase (sGC) and the subsequent generation of cGMP, which in turn, by activating protein kinase (PK)G, serves as a final modulator of vascular relaxation and platelet aggregation [74]. N-3 PUFAs have been shown to increase endothelium-dependent vasodilation in coronaries arteries of normal pigs [75] and in isolated rat aortic ring preparations by enhancing the release of NO [76]. Our current data now suggest an additional interference by DHA in the NO-sGC-cGMP axis. Among the most cited genes up-regulated by IL-1β and down-regulated by DHA, we found the one encoding for PDE5. This enzyme selectively catalyzes the hydrolysis of cGMP into GMP, thus curtailing NO signaling [77]. The importance of cGMP in NO signaling has encouraged, in the past years, the development of PDE5 inhibitors, the first one being sildenafil [78]. Nowadays, PDE5 inhibitors have approved indications for their use in erectile dysfunction and pulmonary hypertension, and have a potential in the treatment of pathological states featuring endothelial dysfunction, including diabetes and the metabolic syndrome, heart failure and Raynaud’s phenomenon [79]. DHA appears in many aspects to reproduce the therapeutic potential of PDE5 inhibitors, down-regulating PDE5 mRNA levels induced by IL-1β. To the best of our knowledge, no previous report had described activation of the PDE5 gene expression by IL-1β, or the related down-regulation by n-3 PUFAs. The presence of Sp1, AP-1 and AP-2 binding sites in the promoter sequence of PDE5 [80] may account for both PDE5 mRNA induction by IL-1β and the modulatory activity exerted by DHA [81, 82].

In conclusion, the data presented here yield novel information on cardiovascular health effects linked to n-3 PUFAs and fish oil consumption. They demonstrate that exposure of human endothelial cells to DHA causes deep changes in gene expression that may prevent endothelial dysfunction and protect the endothelium from the occurrence of atherosclerosis under basal and, more relevant, under pro-inflammatory conditions. Several pathways here shown to be regulated by DHA are new, and suggest promising reinterpretations of the
therapeutic potential of n-3 PUFAs and of their role in modulating activity and function of EPC and stem cells. Being ours, to the best of our knowledge, the first report in the specific endothelial research area, we were unable to produce comparative evaluations with similar literature reports. However our data point out to a global interference by DHA with the expression of NF-κB target genes, as well as with modulators of such activation, confirming and expanding the anti-inflammatory and anti-atherogenic activities highlighted in vivo [21, 23, 25, 26] and providing new molecular explanations to evaluate the role of fish oil-derived n-3 PUFAs in the context of diseases featuring endothelial dysfunction.

Supporting Information

S1 File. Supporting materials and methods.

S2 File. Supporting Tables.

S1 Fig. The most significant network regulated by DHA in resting unactivated conditions. Networks of genes were algorithmically generated with the IPA software based on their connectivity and assigned a score. The intensity of the node color indicates the degree of up- (red) or down-(green) regulation. A continuous line means a direct relationship between the two genes, whereas a discontinuous line indicates an indirect association. The network depicted includes 22 focus molecules.

S2 Fig. Top ten signaling and metabolic pathways regulated by IL-1β as compared to unstimulated conditions. For the functional categorization of genes, Fischer’s exact test was used to calculate a P value (shown as bars) indicating the probability that each biological function assigned to the network is due to chance alone. The ratio (shown as squares) represents the number of differentially expressed genes in a given pathway divided by total number of genes that make up that canonical pathway.

S3 Fig. The most significant network regulated by DHA in IL-1β stimulated conditions. Networks of genes were algorithmically generated with the IPA software based on their connectivity and assigned a score. The intensity of the node color indicates the degree of up- (red) or down-(green) regulation. A continuous line means a direct relationship between the two genes, whereas a discontinuous line indicates an indirect association. The network depicted includes 18 focus molecules.

S4 Fig. siRNA-mediated CD47 and CARD11 knockdown. HUVECs were transfected with non-silencing (siControl), CD47 or CARD11 siRNA for 72 before IL-1β stimulation. (A) Phase contrast images of the endothelial monolayers after silencing of CD47 and CAR11. Bars, 20 μm. (B) Effect of Cd47 and CARD11 silencing on endothelial vitality. After gene silencing and IL-1β stimulation MTT assay was performed. In no culture condition tested were highlighted evidence of toxicity by gene silencing. Absorbance data are expressed as milliunits (mU), mean ± standard deviation (S.D.) (n = 16). (C) HUVEC were transfected with CD47 siRNA, CARD11 siRNA or non-silencing siRNA (siControl) for 72 h before 3 h stimulation with 5 ng/mL IL-1β. CD47 and CARD11 mRNA expression levels were analyzed by qRT-PCR. Data are expressed as fold induction over unstimulated siControl and derive from three independent experiments performed in duplicate. ** < P<0.01 between groups joined by the
S5 Fig. CARD11 functional analysis. HUVEC were transfected with CARD11 siRNA or non-silencing siRNA (siControl) for 72 hours. After 3 h stimulation with 5ng/mL IL-1β total RNA was isolated and gene expression profile of the indicated genes assessed by qRT-PCR. Data are presented as fold induction over siControl + IL-1β and derive from two independent experiments performed in duplicate. *P<0.01 vs siControl + IL-1β.

S6 Fig. CD47 functional analysis. HUVEC were transfected with CD47 siRNA or non-silencing siRNA (siControl) for 72 hours. After 3 h stimulation with 5 ng/mL IL-1β total RNA was isolated and gene expression profiles of the indicated genes assessed by qRT-PCR. Data are presented as fold induction over siControl + IL-1β and derive from two independent experiments performed in duplicate. *P<0.05 vs siControl + IL-1β; **P<0.0 vs siControl + IL-1β.

S7 Fig. Validation of gene expression changes in HUVECs treated with DHA before IL-1β stimulation by qRT-PCR. HUVECs were treated with/without DHA for 48 h and then stimulated with IL-1β. After 3 h mRNA level of various genes was assayed by qRT-PCR. Data are expressed as fold induction over unstimulated control and derive from three independent experiments performed in duplicate. *P<0.05 vs IL-1β alone; **P<0.01 vs IL-1β alone. PDE5α, phosphodiesterase 5α; TGF-β2, transforming growth factor-β2; CARD11, caspase recruitment domain-11; COX-2, cyclooxygenase-2; VCAM-1, vascular cell adhesion molecule-1; ICAM-1, intercellular adhesion molecule-1; E-Sel, E-selectin; MCP-1, monocyte chemoattractant protein-1.

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Author Contributions
Conceived and designed the experiments: MM ES RDC. Performed the experiments: MM TB RM ES MP NC MAC. Analyzed the data: MM VG RM LS CS. Contributed reagents/materials/analysis tools: TB RM LS RDC CS MAC. Wrote the paper: MM ES RDC. Revised the article: RDC. Approved the final manuscript: MM TB RM ES MP NC MAC RDC VG LS CS.
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